

MULTIPLE FORMS OF SOMATOSTATIN IN RAT AND HUMAN BRAIN

by

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TO MY FAMILY

## ABSTRACT

The techniques of high performance liquid chromatography and radioimmunoassay were used to demonstrate that four forms of somatostatin-like immunoreactivity (SSLI) are present in acid extracts of rat brain: somatostatin-14 (SS14), somatostatin 28 (SS28) and 6,000 and 10,000 mol. wt. forms (high molecular weight somatostatin: HMW-SS). In the hypothalamus and amygdala, SS14 was the predominant form of SSLI, but in the median eminence, SS14 and SS28 were present in almost equimolar amounts.

When hypothalamic slices were perfused in vitro with a modified Krebs bicarbonate medium and exposed to a depolarising stimulus of 50mM  $K^+$ , the rate of release of SS14 was significantly increased but no change in the rate of release of SS28 was observed. In contrast, depolarisation increased the rates of release of both SS14 and SS28 from the isolated median eminence. SS28 has been shown by others to be more potent than SS14 in inhibiting growth hormone release from pituitary cells in vitro. The localised abundance of SS28 in the nerve terminals of the median eminence suggests a specific role for this peptide in the hypothalamic regulation of growth hormone secretion.

Many of the neuropathological and biochemical changes characteristic of Alzheimer-type Dementia (ATD) are also observed in cases of Down's syndrome (DS) aged over 40. SSLI is reduced in post-mortem brain from cases of ATD, and to determine if this deficit also occurs in DS, the concentrations of SSLI in temporal cortex from cases of DS and ATD were compared with those in age-matched control subjects. Significant reductions in SSLI were observed only in cases of ATD aged under 70 at death (pre-senile ATD). The molecular forms of SSLI in human post-mortem brain were similar to those found in rat brain. If the depletion of cortical SSLI seen in pre-senile ATD is the result of a defect in the biosynthesis or processing of the peptide, a change in the relative proportions of the various forms of SSLI should be observed. Chromatographic examination of SSLI from cases of pre-senile ATD revealed a disproportionate loss of HMW-SS. In DS, HMW-SS was also significantly reduced although no overall loss of SSLI was seen. In older cases of ATD, the proportions of multiple forms of SSLI did not differ from those in age-matched controls. These findings suggest that pre-senile ATD and DS may share a common abnormality in the biosynthesis and/or post-translational processing of cortical somatostatin.

I declare that the studies presented in this thesis are the result of my own independent investigation with the exception of:

1. Total SSLI release from hypothalamic slices (Chapter 4) was performed by Ms. L.A. Tannahill (MRC Brain Metabolism Unit)
2. Human post-mortem samples (Chapter 5) were dissected by Dr. C.M. Yates and Mr. J. Simpson (MRC Brain Metabolism Unit). Neuropathological assessments were performed by Dr. A. Gordon (Department of Pathology, Western General Hospital). Protein and ChAT assays were carried out by Mr. J. Simpson.
3. Septal lesions, protein and ChAT assays (Chapter 6) were performed by Mr. J. Simpson (MRC Brain Metabolism Unit).

This work has not been and is not being concurrently submitted for any other degree.



Some of the results presented in this thesis have been published as follows:

1. Arbuthnott, G.W., Harmar, A.J., Pierotti, A.R. & Tannahill, L.  
Release of two molecular forms of somatostatin from the rat hypothalamus in vitro. J. Physiol. 349, 34P (1984).
2. Harmar, A.J. & Pierotti, A.R.  
The pattern of molecular forms of somatostatin released by the rat median eminence differs from that released from the hypothalamus as a whole. J. Physiol. 357, 95P (1984).
3. Pierotti, A.R. & Harmar, A.J.  
Multiple forms of somatostatin-like immunoreactivity in the hypothalamus and amygdala of the rat: selective localization of somatostatin-28 in the median eminence. J. Endocrinol. 105, 383-389 (1985).
4. Pierotti, A.R., Harmar, A.J., Tannahill, L. & Arbuthnott, G.W.  
Different patterns of molecular forms of somatostatin are released by the rat median eminence and hypothalamus. Neurosci. Lett. 57, 215-220 (1985).
5. Pierotti, A.R., Harmar, A.J., Simpson, J. & Yates, C.M.  
High Molecular weight forms of somatostatin are reduced in Alzheimer's disease and Down's syndrome. Neurosci. Lett. 63, 141-146 (1986).
6. Pierotti, A.R. & Simpson, J.  
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## References

## CHAPTER 1

### GENERAL INTRODUCTION

## 1.1 INTRODUCTION

The hypothalamus regulates the function of the anterior pituitary gland through the production of a number of chemical messengers. These are synthesized in neurones within the hypothalamus and transported to the median eminence. Here they are stored in nerve terminals and released into a network of portal capillaries to reach the pituitary gland and influence the synthesis and secretion of anterior pituitary hormones. (For reviews see Harris 1948, Harris 1955). The first evidence for the existence of a factor which inhibited the release of growth hormone (GH) was produced in 1968: Krulich, Dhariwal & McCann showed that a fraction derived from hypothalamic extracts inhibited GH release from rat pituitary incubates in vitro. They postulated that GH secretion was regulated by two factors – one stimulatory and one inhibitory. It was a further five years before Brazeau et al. (1973) were able to isolate and sequence the GH release inhibitory factor – the cyclic tetradecapeptide somatostatin. The tetradecapeptide contains a disulphide bridge linking cysteine residues at positions 4 and 14 (figure 1.1.1).

The term somatostatin was first applied to the cyclic tetradecapeptide, however, subsequent work has shown that somatostatin-like peptides constitute a family of related molecules: the tetradecapeptide somatostatin (somatostatin 14: SS14), an N-terminally extended form (somatostatin 28: SS28, Pradyrol et al 1980) and one or more larger forms (Spiess & Vale, 1980; Patel et al., 1981; Benoit et al., 1982b), all of which arise from a common precursor (proSS) of molecular weight 10,400 (Shen et al., 1982; Goodman et al., 1983).

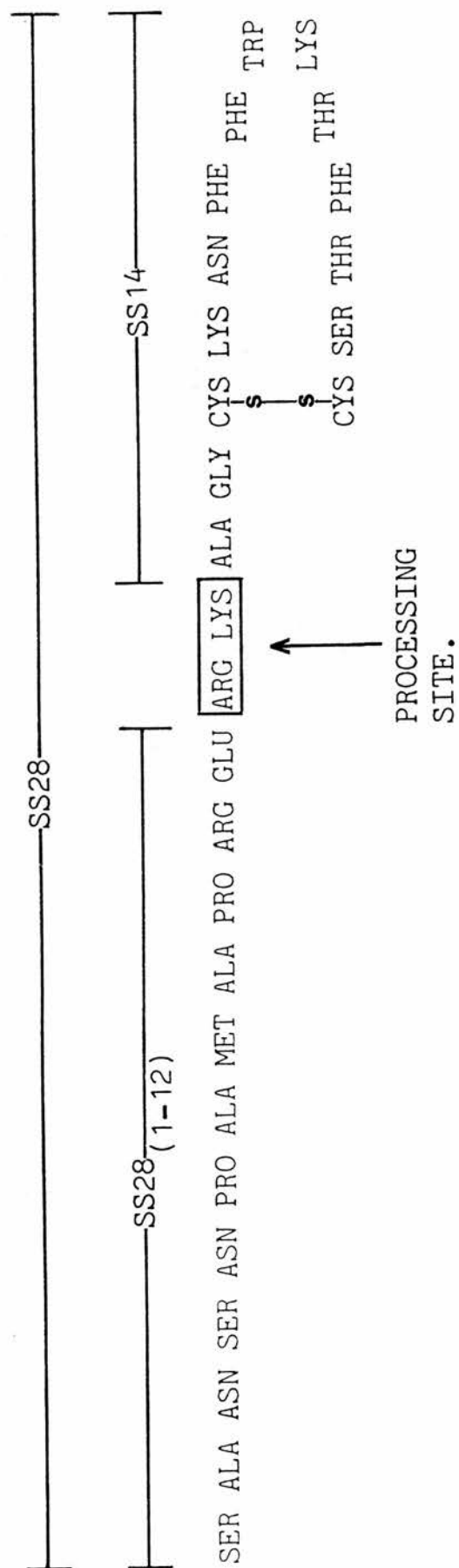


Figure 1.1.1 The amino acid sequences of SS28, SS14 and SS28(1-12)

The pair of basic amino acids where SS28 is cleaved into SS14 and SS28(1-12) is marked processing site. The disulphide bridge between Cys residues 4 and 14 of the SS14 sequence is indicated.

In addition to inhibiting GH secretion, the peptide was found to inhibit the secretion of thyrotropin (TSH) from the pituitary gland (Vale et al., 1975) and the release of glucagon and insulin from the pancreas (Koerker et al., 1974). Radioimmunoassay (Arimura et al., 1975; Brownstein et al., 1975) and immunocytochemical (Hokfelt et al., 1975a) studies showed that somatostatin-like immunoreactivity (SSLI) was not confined to the hypothalamus but was distributed widely in the central and peripheral nervous system and in a number of endocrine cell types, including the D cells of pancreatic islets and the D cells of the digestive tract. In addition to its role as a neurohormone in the regulation of pituitary GH secretion, the peptide has been suggested to function as a neurotransmitter within the nervous system and as a "paracrine" factor secreted from one group of endocrine cells and exerting its influence on neighbouring cells.

## 1.2 DISTRIBUTION

### 1.2.1 Hypothalamus and preoptic area

The highest concentration of SSLI in the nervous system is found in nerve terminals adjacent to the primary capillaries of the hypophysial portal blood vessels in the median eminence (Johansson et al., 1984; Krisch, 1978). These nerve terminals have their origin in a concentrated group of somatostatin-producing cell bodies which lie close to the third ventricle and extend from the preoptic area to the rostral pole of the median eminence, where they merge with the parvocellular portions of the paraventricular nucleus. The axons of these neurons have been postulated to travel to the



median eminence along two pathways, one following a direct route along the wall of the third ventricle and the other performing a lateral loop, entering the median eminence by way of the medial forebrain bundle (Epelbaum et al., 1981; Palkovits et al., 1980). The relative contributions of these two pathways to the innervation of the median eminence remains controversial. Periventricular somatostatinergic neurones project to a number of regions of the brain outside the hypothalamus (Krisch, 1978), including the organum vasculosum laminae terminalis, the subfornical organ and other neurohaemal structures. These neurones also project to a number of areas in the limbic system, including the septum, stria terminalis, olfactory tubercle, amygdala, hippocampus and habenula, as well as to the posterior pituitary gland (Palkovits et al., 1982).

Somatostatin-containing cell bodies are abundant in a number of other hypothalamic nuclei, notably the arcuate, ventromedial and suprachiasmatic nuclei. These cells are thought to be involved in fibre networks largely confined to their nuclei of origin, and in them somatostatin presumably subserves neurotransmitter rather than neuroendocrine functions.

#### 1.2.2 Extrahypothalamic brain

Somatostatin-containing neuronal perikarya are widely distributed in the rest of the brain. SSLI is present in all areas of the cerebral cortex, predominantly in laminae II, III and VI (Bennett-Clarke et al., 1980). Although concentrations of the peptide in cortex are relatively modest, the size of the cortex implies that it contains more SSLI than any other region of the brain. Large numbers of somatostatin-containing neurones are also

present in the amygdala, hippocampus and striatum (Krisch, 1978).

In each of these regions, most somatostatin neurons are intrinsic to their structures of origin and are thought to function predominantly as interneurons. However, a somatostatinergic projection from the amygdala to the lower brain stem has been described (Kawai et al., 1982).

Somatostatin is present in large amounts in most sensory pathways. In the auditory system, SSLI is detected in the cochlear nuclei, superior olivary complex, lemniscal nucleus, inferior colliculus and medial geniculate body (Takatsuki et al., 1981). SSLI is seen throughout the visual pathway (Finley et al., 1981; Krisch & Leonhardt, 1979) and olfactory pathway (Johansson et al., 1984).

Somatostatin may function as a sensory neurotransmitter in a population of dorsal root ganglion neurones distinct from those containing other neuropeptides (Hokfelt et al., 1975b). These neurones have terminals in the substantia gelatinosa of spinal cord (predominantly lamina II) (Hokfelt et al., 1976). SSLI is present in the peripheral processes of these neurones in the skin and other structures but no function has yet been ascribed to it. Descending and ascending somatostatin-containing pathways within the spinal cord and a somatostatin-containing projection from the periaqueductal grey to the nucleus raphe magnus may play a role in the modulation of sensory input to the central nervous system (CNS) (Beitz et al., 1983).

In all areas of the digestive tract except the stomach, somatostatin-containing neurones are abundant (Keast et al., 1984). Cell bodies are present in the submucosal plexus and in smaller

numbers in the myenteric plexus. Nerve terminals are found predominantly in the mucosal and sub-mucosal layers. Some of these fibres derive from neurones intrinsic to the gut but a proportion contain noradrenaline and originate in the autonomic ganglia (Schultzberg et al., 1980; Costa & Furness, 1984).

### 1.2.3 Distribution in non-neuronal tissues

As soon as antibodies to synthetic SS14 became available, immunocytochemical techniques revealed that the peptide was present in a number of endocrine cell types in concentrations comparable to those in the hypothalamus (Hokfelt et al., 1975a). Notably, somatostatin was found to correspond to the factor present in pancreatic islets which inhibited insulin secretion (Hellmann & Lernmark, 1969). SSLI is located in a population of islet cells (D cells) which also stain intensely with silver and are distinct from cells containing insulin (B cells) glucagon (A cells) and pancreatic polypeptide (PP cells). In the digestive tract, SSLI is present in a group of epithelial endocrine cells (D cells). In man, these cells are most abundant in the stomach and decrease in number in a gradient from stomach to lower colon (Keast et al., 1984). These D cells extend microvillar processes into the lumen of the digestive tract and are sensitive to the concentrations of hydrogen ions and nutrients. SSLI secreted from intestinal D cells may play a role in regulating the release of other gut hormones, acid secretion, nutrient absorption and intestinal motility. SSLI originating in the gastro-intestinal tract also enters the circulation and may play a role in the control of pancreatic hormone release. SSLI is present in some of the calcitonin-producing parafollicular cells (C

cells) in the thyroid (Van Noorden et al., 1977), where the peptide may regulate the secretion of calcitonin. D-type cells in the salivary glands also contain SSLI (Schusdziarra et al., 1978).

#### 1.2.4 Co-existence with other transmitters

SSLI has been shown to co-exist with other transmitters in neurones in several regions of the nervous system. In the cortex of the rat, monkey and human, a significant number of neurones contain both SSLI and neuropeptide Y (NPY) immunoreactivity (Chronwall et al., 1984; Hendry et al., 1984). In the cat hippocampus and visual cortex, all neurones exhibiting SSLI also contain GABA, although only a small proportion of cortical GABA neurones contain SSLI (Somogyi et al., 1984). Co-existence of SSLI and GABA has also been observed in the nucleus reticularis thalami. In the striatum, all somatostatinergic neurones also exhibit NPY immunoreactivity and in addition stain histochemically for NADPH diaphorase, an enzyme of restricted distribution in the CNS but of unknown function (Vincent et al., 1983). In the periphery, a proportion of the noradrenergic neurones in certain sympathetic ganglia have been shown to contain SSLI (Costa & Furness 1984). For example, the submucous plexus and mucosa of the intestine are innervated by noradrenergic neurones of the coeliac-mesenteric ganglia where 25% of the noradrenergic cells contain SSLI. The acetylcholine neurones of the submucosa of the small intestine can be divided into three sub-groups: those that have immunoreactivity to substance P, those that have immunoreactivity for somatostatin, cholecystikinin (CCK) and NPY, and those in which no additional marker substance has been

demonstrated (Schultzberg et al., 1980).

### 1.3 BIOSYNTHESIS AND POST-TRANSLATIONAL PROCESSING OF PROSOMATOSTATIN

#### 1.3.1 Biosynthesis

Peptidergic neurones are nerve cells specialized in the synthesis and secretion of neuropeptides. Various studies have shown that small peptide neurotransmitters and hormones are synthesised as part of larger precursor polypeptides (prohormones) by a series of sequential proteolytic cleavages. The initial neuropeptide gene product synthesised on the rough endoplasmic reticulum is the pre-pro-protein. Pre-pro-proteins consist of a "pre" or "signal" sequence at the N-terminus which is composed of between 15-30 amino acids rich in hydrophobic residues. The signal sequence promotes the attachment of the nascent prohormone to the membrane of the rough endoplasmic reticulum and its translocation through the membrane into the intracisternal space. The signal peptide is cleaved from the protein before the completion of translation to generate the prohormone. The prohormone is then translocated from the rough endoplasmic reticulum into the Golgi apparatus where it is packaged into secretory granules. Some prohormones undergo their first proteolytic cleavage in the Golgi apparatus and the final steps of proteolytic processing occur after packaging into secretory granules. However, other prohormones are cleaved after packaging into secretory granules.

Recombinant DNA techniques have been used to determine the nucleotide sequences of messenger RNAs encoding these precursors

(from which the amino acid sequences of the precursors may be predicted) and of the DNA sequences corresponding to their respective genes. Although there is evidence that lower vertebrates may possess multiple somatostatin genes (Hobart et al., 1980), in mammals, only a single gene is present (Shen & Rutter, 1984; Montminy et al., 1984) which in man is located on chromosome 3 (Naylor et al., 1983). After transcription, mature somatostatin-mRNA (SS-mRNA) is generated from a larger precursor RNA by the splicing out of an intervening sequence of 600-900 base pairs which interrupts the coding region of the SS-mRNA.

In both rat and human tissues, the primary product of the translation of SS-mRNA is preprosomatostatin (preproSS), a polypeptide of 116 amino acids with the sequence of SS14 located at the C-terminus (figure 1.3.1: Shen et al., 1982; Goodman et al., 1983). There is a high degree of conservation between the rat and human preproSS sequences. The two proteins differ by only four amino acids, two of which are in the signal sequence. The sequence of 24 amino acids at the N-terminus of preproSS constitutes the signal peptide. This peptide is cleaved from the prohormone to generate prosomatostatin (proSS), a 92 amino acid peptide (mol. wt. 10,400), the largest form of SSLI present in significant quantities in tissues.

### 1.3.2 Post-translational processing

After translation, a prohormone is modified or processed in a number of ways to produce biologically active peptides. The protein is cleaved into smaller active fragments by a special group of intracellular proteases located in the Golgi apparatus and

[illegible]

The rat sequence is shown: amino acids which differ in humans are shown above the main sequence. Regions corresponding to the signal peptide, SS28 and SS14 are indicated. Sequences taken from Goodman et al. (1983: rat) and Shen et al. (1982: human).

developing secretory granules. Other post-translational modifications such as glycosylation, amidation and acetylation also occur in the Golgi apparatus and secretory granule. For reviews of processing see Mains et al., 1983; Loh et al., 1984; Docherty & Steiner, 1982; Douglass et al., 1984.

Virtually all of the prohormones have pairs of basic amino acids flanking the neuropeptide sequences that are to be cleaved. The most common pair of basic residues is the Lys-Arg sequence although Arg-Lys, Arg-Arg and Lys-Lys can also be found. This structural pattern suggests that prohormone converting enzymes exhibit a specificity for pairs of basic amino acid residues. However, not all proteolytic cleavages involved in precursor processing occur at pairs of basic amino acids. Some cleavages occur immediately C-terminal to a single basic amino acid, usually arginine. Additionally, a number of cleavages which do not involve lysyl or arginyl residues have been observed.

The exact nature of these processing enzymes is not clear but both serine and thiol proteases have been implicated in prohormone conversion. In order to cleave prohormones at pairs of basic amino acids, two types of proteolytic activity are necessary. The first is an enzyme which would recognise the paired basic residues flanking the peptide sequences to be cleaved. This activity is often referred to as "trypsin-like" although it is clear that many of these enzymes have properties quite unlike trypsin (Loh et al., 1984). A similar "trypsin-like" enzyme may operate at single arginyl residues, although an enzyme with a specificity towards single basic residues has not been identified. The second activity is carboxypeptidase-B-like and would remove the basic residues from

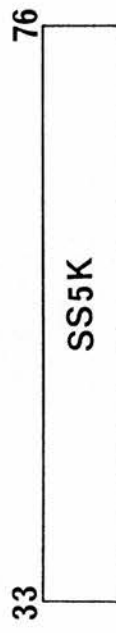
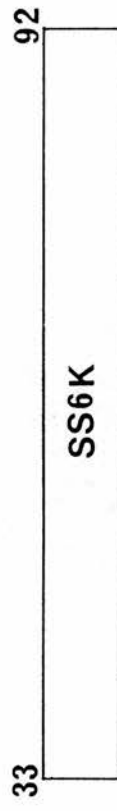
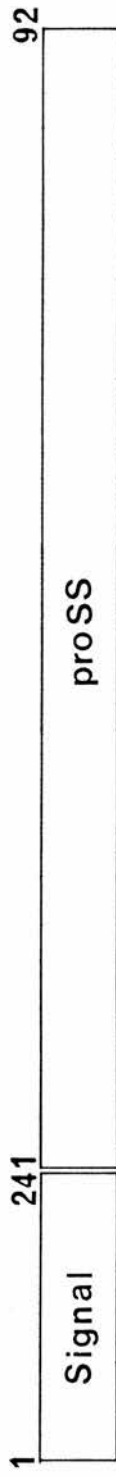
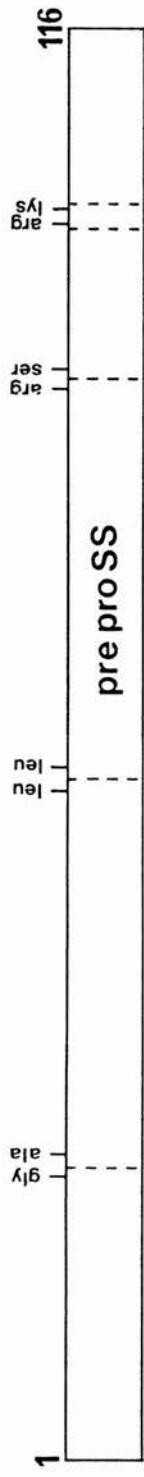


the C-terminus of the cleaved peptide.

Not all basic residues serve as cleavage sites for processing enzymes. Although pairs of basic amino acids are generally the signal for proteolytic cleavage, not all such pairs are cleaved. Single basic amino acids are only cleaved in a few specific cases. Geisow & Smyth (1980) have proposed that certain pairs of basic amino acid residues are not cleaved in some prohormones because they are found in the  $\alpha$ -helix structure of the protein. Those parts of a prohormone sequence with no known biological function may be involved in folding the prohormone to provide the appropriate conformation for specific cleavage.

The work of Benoit et al. (1982b) suggests that there are three cleavage sites within the proSS sequence and that at least seven different peptides are generated from the precursor (figure 1.3.2). Somatostatin 28 (SS28), an octacosapeptide isolated and sequenced from porcine intestine (Pradyrol et al., 1980) was the first N-terminally extended form of SS14 to be identified. Both SS28 and somatostatin 25 (SS25: a pentacosapeptide identical to SS28 except the first 3 residues at the N-terminus are deleted), were also purified from ovine hypothalamus (Esch et al., 1980). However, it has been suggested that SS25 was generated artefactually from SS28 during the extraction procedure (Esch et al., 1980). Two larger peptides which contain the SS14 sequence have been identified using gel filtration chromatography (Benoit et al., 1982b): a 6,000-7,500 mol. wt. form (SS6k) and a 10,000 mol. wt. species (SS10k), thought to correspond to the intact prohormone. A 32 amino acid peptide corresponding to residues 1-32 of proSS (proSS<sub>1-32</sub>: Schmidt et





al., 1985) has been isolated from porcine intestine. This peptide would be generated after conversion of SS10k into SS6k.

SS28 contains a pair of basic amino acid residues adjacent to the SS14 sequence which is known to be a potential cleavage site for processing enzymes. A fragment consisting of the first twelve amino acids of SS28 (SS28<sub>(1-12)</sub>) is generated after conversion of SS28 to SS14. Benoit et al. (1982a) have shown the presence in rat hypothalamus and pancreas of a dodecapeptide with the identical amino acid composition to synthetic SS28<sub>(1-12)</sub> and have subsequently shown the widespread distribution of this peptide in the rat (Benoit et al. 1982c). Using a RIA directed towards the C-terminus of the SS28<sub>(1-12)</sub> sequence, Benoit et al. (1982b) have shown that there are three forms of SS28<sub>(1-12)</sub> LI in rat brain: SS28<sub>(1-12)</sub> itself and 5,000 (SS5k) and 8,000 (SS8k) species. SS8k has subsequently been shown to correspond to the prohormone without the SS14 sequence and SS5k, a possible intermediate in the biosynthesis of SS14, corresponding to the 44 amino acids at the C-terminus of SS8k (Benoit et al., 1984).

All of the peptides produced from proSS are generated by cleavage of proSS at three sites:

- i) cleavage at the dibasic pair Arg<sub>77</sub>-Lys<sub>78</sub>
- ii) cleavage at the C-terminal side of Arg<sub>64</sub>
- iii) cleavage between Leu<sub>32</sub>-Leu<sub>33</sub>.

The sequence of SS14 is immediately preceded by Arg-Lys, a pair of basic amino acids which is one of the classical sites for post-translational processing. An "SS28 convertase" with a molecular weight of around 90,000 has been described (Gluschankof et al., 1984). This enzyme will convert SS28 into SS14, SS28<sub>(1-12)</sub>

arginine and lysine (Gluschankof et al., 1985) but does not appear to cleave proSS directly into SS14. This converting activity is located in the secretory granule membrane in the rat cortex (Gomez et al., 1985) and presumably consists of "trypsin-like", "carboxypeptidase-like" and possibly "amino peptidase-like" components. At present the possibility that the "SS28 convertase" consists of more than one enzyme cannot be ruled out.

It is likely that a different processing enzyme is responsible for the generation of SS28 from proSS. The sequence Leu-Gln-Arg-Ser immediately to the N-terminus of SS28 is a single basic amino acid cleavage site and may be the substrate for a processing enzyme of novel specificity. The generation of SS6k and proSS<sub>1-32</sub> from SS10k occurs at a pair of leucyl residues. Such a cleavage site is not common but cleavage at paired leucyl residues has been reported in the formation of angiotensin I (Skeggs et al., 1957).

#### 1.4 CONTROL OF HYPOTHALAMIC SOMATOSTATIN RELEASE

There is substantial evidence that the neurosecretory somatostatin-containing neurones in the hypothalamus receive a catecholaminergic (probably dopaminergic) input (see Arimura & Fishback, 1981). Thus, in rats, intraventricular dopamine stimulates secretion of SSLI and inhibits the release of GH (Maeda & Frohman 1980), whereas dopamine antagonists such as chlorpromazine and pimozide increase circulating levels of GH. However, circulating GH levels were increased following peripheral administration of L-DOPA or of dopaminergic agonists and reduced after administration of the tyrosine hydroxylase inhibitor  $\alpha$ -methyl

p-tyrosine (Maeda & Frohman 1980). These paradoxical effects suggest that both somatostatin and growth hormone releasing hormone (GHRH) may be regulated by dopaminergic stimuli and that the effects of dopaminergic agents on pituitary GH secretion may reflect a delicate balance between effects on the release of these two regulatory factors. Growth hormone can exert negative feedback on its own secretion both directly, by stimulating the release of SSLI from the hypothalamus (Chihara et al., 1981; Sheppard et al., 1978; Berelowitz et al 1981a) and indirectly by stimulation of hepatic somatomedin C synthesis (Abe et al., 1983). Somatomedin C has been shown to stimulate release of hypothalamic SSLI (Berelowitz et al., 1981b) and to inhibit GH secretion (Abe et al., 1983).

In addition to its role in the regulation of pituitary GH secretion, somatostatin has been shown to be a potent inhibitor of TSH secretion (Vale et al., 1975). Dopaminergic agonists have been shown to inhibit and dopamine antagonists to stimulate TSH secretion in vivo: these effects are presumably mediated by the dopaminergic control of hypothalamic somatostatin release (Krulich et al., 1977). Thyroid hormones inhibit the secretion of SSLI (Berelowitz et al., 1980) and this may contribute to the feedback pathway by which TSH regulates its own release.

Several peptides have been shown to modulate the release of SSLI from the hypothalamus. Substance P and neurotensin have been shown to stimulate release of SSLI in vitro and to inhibit GH secretion in vivo (Sheppard et al., 1979, Hermansen 1980; Maeda & Frohman, 1980). The effect of substance P on GH release can be abolished by passive immunisation with antisomatostatin serum. Vasoactive intestinal polypeptide (VIP) inhibits release of SSLI in vitro and

stimulates GH release in vivo (Epelbaum et al., 1979) and glucagon has been shown to stimulate the secretion of somatostatin in vitro (Shimatsu et al., 1981) and in vivo (Abe et al., 1978).

## 1.5 ACTIONS OF SOMATOSTATIN

### 1.5.1 Actions on endocrine cells

The actions of somatostatin upon physiological processes are widespread and almost exclusively inhibitory (Table 1.1) (see Gerich 1981 for review). In the pituitary gland, somatostatin inhibits the secretion of GH both in basal conditions and in response to a wide range of stimuli, including arginine, exercise and insulin-induced hypoglycaemia. In man, episodic surges of GH release are suppressed. Both basal and thyrotropin releasing hormone (TRH)-stimulated secretion of TSH are inhibited by somatostatin (Vale et al., 1975; Hirooka et al., 1978). Although release of prolactin, adrenocorticotrophic hormone (ACTH) and the gonadotropins are unaffected by somatostatin in normal human subjects, the peptide may influence the secretion of ACTH in Addison's Disease and Nelson's syndrome, and depress the elevated levels of prolactin found in some acromegalics (Gerich, 1981).

Somatostatin inhibits the secretion of all three peptide hormones with which it is associated in pancreatic islets (Koerker et al., 1974). Both basal insulin release and secretion in response to a variety of stimuli (glucose, arginine, glucagon and food) is inhibited. Both basal and stimulated glucagon release is inhibited by somatostatin. In the exocrine pancreas, secretion of bicarbonate, water and of digestive enzymes is inhibited.

TABLE 1.1

## INHIBITORY ACTIONS OF SOMATOSTATIN

Tissue	Response
Pituitary	Secretion of growth hormone and thyroid-stimulating hormone
Pancreas (Islets)	Secretion of Insulin, Glucagon, and pancreatic polypeptide
Pancreas (Exocrine)	Secretion of $\text{HCO}_3$ , $\text{H}_2\text{O}$ , enzymes
Stomach	acid secretion, gastric emptying
Intestine	Amine and peptide hormone secretion, absorption of triglycerides, sugars, amino acids and other nutrients, mesenteric blood flow.
Gall Bladder	bile flow
Kidney	Renin secretion, aldosterone response to angiotensin II
Parathyroid	Parathyroid hormone secretion
Salivary Gland	Secretion (Salivary flow)
Blood platelets	Aggregation
Neurones	Inhibition or excitation of firing
Thyroid	Calcitonin secretion.



In the digestive tract, a wide variety of functions are inhibited by somatostatin (see Reichlin 1981; Arnold & Lankisch, 1980; Gerich, 1981; Sundler et al., 1980). These include the secretion of many hormones and neurotransmitters, inhibition of exocrine secretion by the stomach, small intestine and pancreas, motility of the stomach, gall bladder and perhaps small intestine, decreased intestinal absorption of food and water, intestinal blood flow and proliferation of stomach mucosal cells. These effects are likely to be predominantly paracrine in nature, due to the diffusion of the peptide released locally to affect other cells in the same area. There may, however, be a significant contribution from blood-borne SSLI in a classic hormone action and also by intraluminal secretion into the gut. The concentration of SSLI in the circulation after a meat-based meal is sufficient to inhibit gastric acid secretion and islet cell function.

#### 1.5.2 Effects on neuronal activity

Numerous groups have reported effects of somatostatin on the electrophysiological activity of cortical neurones but the results have been conflicting. Extracellular recording techniques have demonstrated inhibitory responses to somatostatin in rat cerebellum, cerebral cortex, hypothalamus (Renaud et al., 1975) and in those cells in the dorsal horn of the spinal cord in cats which are activated by noxious stimuli (Randic & Miletic, 1978).

Intracellular studies have revealed that hippocampal pyramidal cells, dorsal horn interneurons and cells in the myenteric plexus and in the ciliary ganglion respond to the application of SS14 with a hyperpolarisation associated with a decrease in membrane

resistance (Pittman & Siggins 1981). In the ciliary ganglion, voltage clamp studies suggest that this change in membrane properties may result from an activation of the resting  $K^+$  conductance (Kondo et al., 1982).

Ioffe et al. (1978) found an increased firing rate in 50% of neurones in rabbit sensory motor cortex in response to iontophoretic application of somatostatin. An increase in the rate of firing in approximately 60% of rat cortical neurones was observed by Olpe et al. (1980). Excitatory effects of SS14 have also been described in hippocampus (Dodd & Kelly, 1978). Hippocampal pyramidal cells have been reported to respond to the peptide with strong excitation, associated with membrane depolarisation. In the myenteric plexus, excitatory responses to SS14 are associated with membrane depolarisation and increased membrane resistance, possibly due to the inactivation of a  $K^+$  conductance.

Somatostatin enhances both inhibitory and excitatory postsynaptic potentials in cultured cortical and spinal cord neurones (Dichter & Delfs, 1981; MacDonald & Nowak, 1981), suggesting that in some systems it may act presynaptically to enhance the release of other inhibitory and excitatory transmitters.

### 1.5.3 Selective actions of SS14 and SS28

The knowledge of possible selective actions of SS14 and SS28 on different physiological processes is limited. Studies conducted in vivo are difficult to interpret since they are rarely suited to rigorous pharmacological analysis. The half-life of SS28 in plasma is approximately twice that of SS14 (Polonsky et al., 1982) and this may account for the greater apparent potency of SS28 compared to

SS14 in some tests. SS28 has been reported to inhibit pancreatic insulin release and pituitary growth hormone secretion with a potency some 10 times greater than that of SS14 (Mandarino et al., 1981; Brazeau et al., 1981). In contrast, pancreatic glucagon secretion is 10 fold more sensitive to inhibition by SS14 than by SS28 (Mandarino et al., 1981). These studies suggest that in intact cells there may be 2 discrete populations of receptors specific for SS28 and SS14.

#### 1.5.4 Receptor binding studies

Somatostatin receptors have been identified in brain, pituitary and peripheral tissue homogenates by binding techniques using stable iodinated analogues of SS14 and SS28 (Reubi et al., 1981; Srikant & Patel, 1981a; Epelbaum et al., 1982). Additionally, somatostatin receptors have been mapped in rat and human brain by autoradiographic techniques (Uhl et al., 1985; Leroux et al., 1985). Early experiments on isolated pancreatic and pituitary tissue suggested that there might be two classes of somatostatin receptor exhibiting selectivity for SS28 and SS14 (Srikant & Patel, 1981b). However, binding displacement data and the identical regional distribution of SS14 and SS28 binding sites observed in recent studies (Moyse et al., 1984; Uhl et al., 1985) has provided evidence for only one class of somatostatin receptor to which both SS14 and SS28 analogues bind with high affinity.

Consistent with the role of SS14 and SS28 in the regulation of pancreatic insulin and glucagon release and of pituitary GH release, high concentrations of somatostatin receptors are present in both of these tissues. Within the CNS, the distribution of somatostatin

receptors corresponds closely to the distribution of SSLI. Somatostatin receptors are widely distributed with particularly high concentrations in somatostatin-rich areas (cortex, hippocampus, amygdala and striatum). In the brain stem and cerebellum, where few somatostatin-containing fibres are found, very low numbers of receptors are present.

#### 1.5.5 Mechanism of action

Somatostatin has been proposed to act on target tissues by two, possibly interrelated mechanisms: i) by inhibiting the formation of cyclic 3',5'-AMP (cAMP) and ii) inhibiting calcium entry into the cell. Many neurotransmitters and hormones exert their actions by modulating the activity of the cAMP-synthesising enzyme adenylate cyclase (Rodbell, 1980) (figure 1.5.1). Substances stimulating enzyme activity do so as the result of formation of a complex between their receptor, adenylate cyclase and a stimulatory coupling factor ( $N_A$ ) which possesses GTPase activity. The effects of receptor activation can be mimicked by cholera toxin, which ADP-ribosylates  $N_A$  leading to a long-lasting stimulation of adenylate cyclase. Receptors for substances which inhibit adenylate cyclase are thought to interact with another GTPase known as  $N_I$ , which in turn binds to adenylate cyclase and inhibits enzyme activity.  $N_I$  can be inactivated by islet-activating protein (IAP) from *bordetella pertussis*, which ADP-ribosylates the protein. IAP thus abolishes the inhibitory actions of those hormones which act on adenylate cyclase through this mechanism. In the somatotroph, GHRH and somatostatin appear to act at receptors which bind respectively to  $N_A$  and  $N_I$ . Thus, application of

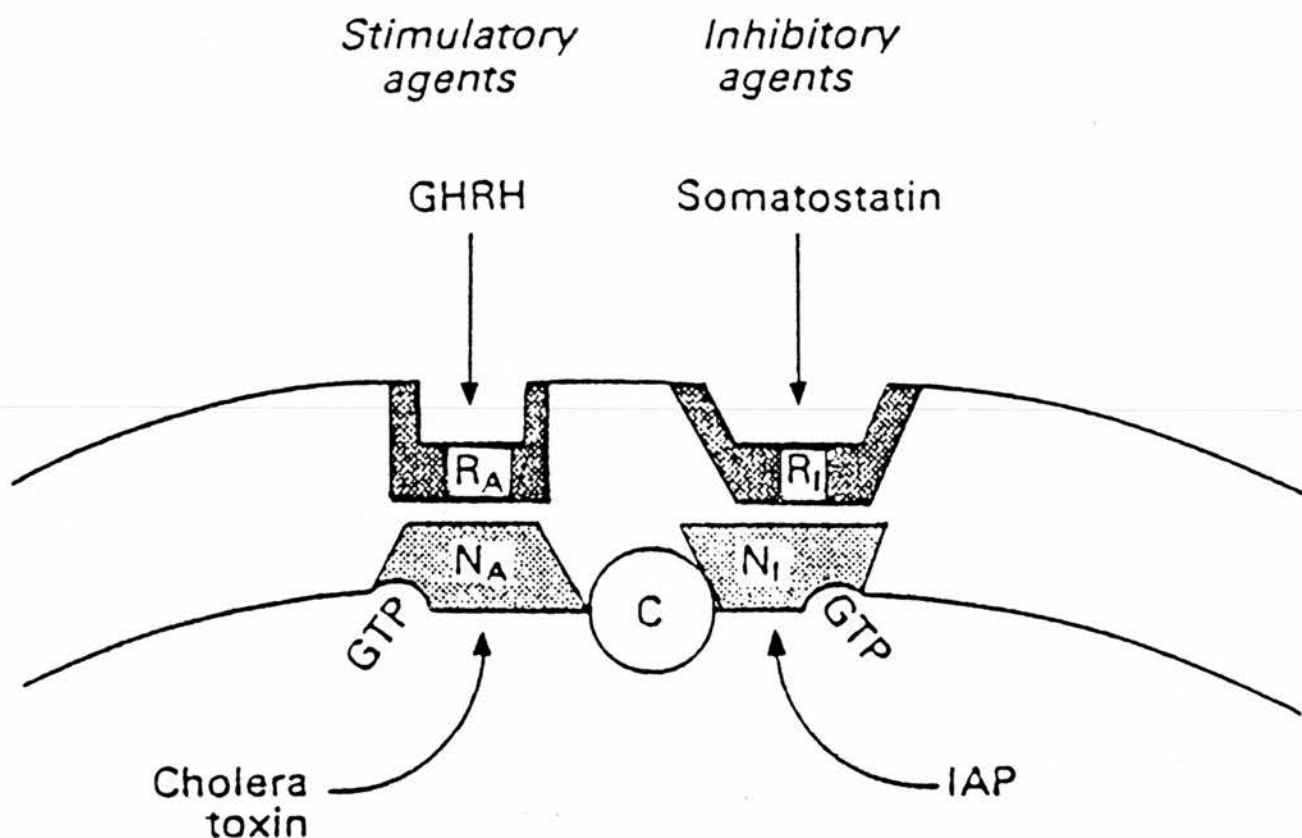


Figure 1.5.1 Mechanisms of action of GHRH and somatostatin upon pituitary cell adenylate cyclase

GHRH receptors ( $R_A$ ) stimulate adenylate cyclase (C) through a stimulatory nucleotide regulatory unit ( $N_A$ ) and somatostatin receptors ( $R_I$ ) inhibit cyclase activity through linkage with an  $N_I$  unit. Both  $N_A$  and  $N_I$  exhibit GTPase activity. Cholera toxin and islet activating protein (IAP) are capable of long-lasting activation of  $N_A$  and  $N_I$  respectively; both effects are due to ADP-ribosylation of the regulatory proteins (adapted from Rodbell, 1980).

GHRH to rat pituitary cells results in a stimulation of both GH secretion and intracellular cAMP content (Bilezikjian and Vale, 1983). Somatostatin inhibits basal and GHRH-stimulated GH secretion and attenuates but does not totally abolish the rise in intracellular cAMP levels in response to GHRH. The effects of somatostatin on GH release and intracellular cAMP accumulation are abolished by IAP, confirming that the peptide exerts its effect on these responses through the  $N_I$  regulatory protein (Cronin et al., 1983). Although these studies point to a role for cAMP in the mechanism of action of somatostatin, in many cell types the peptide is also capable of inhibiting responses to cAMP analogues, suggesting a second site of action "downstream" from adenylate cyclase. The nature of this second target for somatostatin action is not clear.

Electrophysiological studies of the actions of somatostatin on pancreatic islet  $\beta$ -cells (Pace and Tarvin, 1981) suggest that the peptide may increase cellular  $K^+$  permeability, and thus hyperpolarise the cell membrane. The well-documented inhibitory effect of somatostatin upon membrane permeability to calcium (Bhathena et al., 1976; Oliver, 1976) may reflect the response of voltage-sensitive calcium channels to  $K^+$ -mediated membrane hyperpolarisation. The ability of somatostatin to inhibit a wide variety of secretory processes may result from these effects on cellular calcium fluxes, which are of prime importance in the regulation of exocytosis.

#### 1.5.6 Pharmacology of somatostatin analogues

In early studies of the pharmacology of somatostatin analogues,

the effects of single amino acid replacements within the SS14 sequence were assessed. For example, replacement of individual amino acids with alanine generally resulted in analogues with negligible biological activity. However, substitution with alanine at positions 4, 10, 12 or 13 resulted in analogues of limited potency and the Ala<sup>2</sup> and Ala<sup>5</sup> analogues retained the full biological potency of native SS14 (Vale et al., 1978). Similarly, when each amino acid in the SS14 sequence was replaced by its D-isomer, inactive or only slightly active analogues were obtained. Exceptions were [D-Cys<sup>14</sup>]-SS14 (equipotent with native SS14) and [D-Trp<sup>8</sup>]-SS14 which is 6-8 times more active than SS14 (Vale et al., 1978). Although neither of these analogues proved to be longer acting than SS14, the [D-Trp<sup>8</sup>] modification has formed the basis for the development of many analogues with prolonged actions and some selectivity in hormone-releasing action. For example, [p-NH<sub>2</sub>-Phe<sup>4</sup>, D-Trp<sup>8</sup>]-SS14 inhibits GH release 15 times more effectively than SS14 itself but is only slightly more active than SS14 in inhibiting the release of insulin and glucagon (Murphy et al., 1981). Derivatives based on [des-Asn<sup>5</sup>] modification are less potent in inhibiting glucagon release than in inhibiting the release of GH and insulin (Effendic et al., 1975). Analogues such as [des-Ala<sup>1</sup>, Gly<sup>2</sup>, His<sup>4,5</sup>, D-Trp<sup>8</sup>]-SS14 have little activity on insulin secretion compared to their effects on GH and glucagon (Sarantakis et al., 1978) and many active position 8 analogues have much reduced inhibitory effects on gastric acid secretion compared to other secretory functions (Reed et al., 1978).

These studies resulted in the identification of a minimum sequence essential for biological activity (Phe-D-Trp-Lys-Thr,



residues 7-10 of the SS14 sequence) which in native SS14 adopts a  $\beta$ -turn configuration; many potent analogues are based upon modifications of this simplified structure. The prototype analogue of this type is shown in figure 1.5.2b. Two analogues of potential clinical importance have been developed from this structure. SMS 201-995 (figure 1.5.2c: Bauer et al., 1982) is a long-acting analogue which exhibits a selective action on GH secretion. This analogue lasts up to nine hours in plasma following subcutaneous injection. It is about 20 times more potent than native SS14 as an inhibitor of GH secretion and binds well to binding sites in GH-secreting pituitary adenomas. Administration of SMS 201-995 has resulted in a fall of GH in all patients tested. As well as being effective in acromegalics it has been used with significant benefit in patients with vipomas, glucagonomas, gastric ulcer and the vascular complications of late-stage diabetes. It is interesting that although it is more potent than native somatostatin in the inhibition of GH secretion, it is a less potent inhibitor of glucagon.

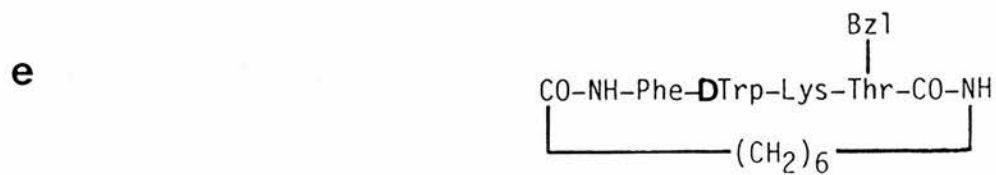
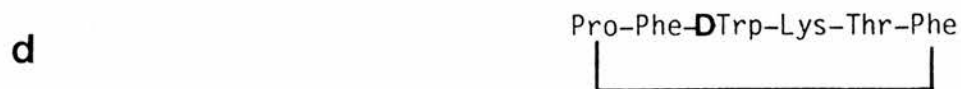
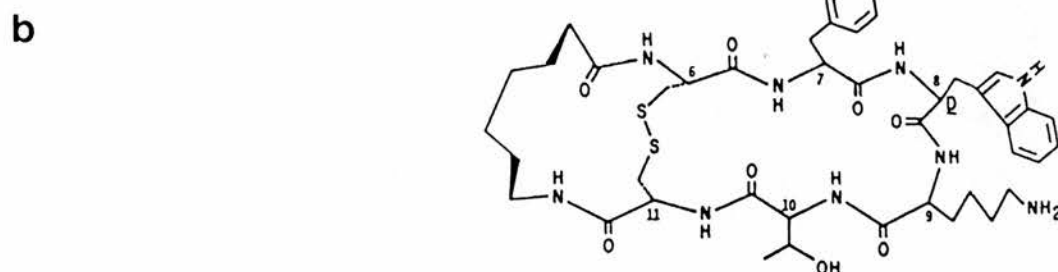
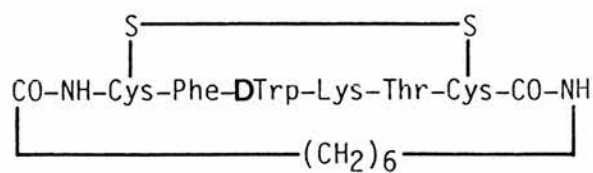
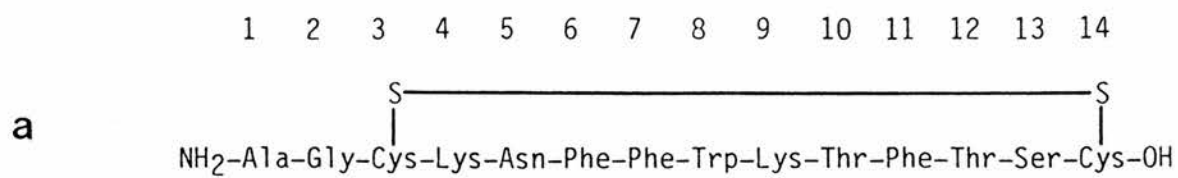
Figure 1.5.2d shows an analogue developed by computer modelling, which is long acting and 20-25 times more potent than SS14 in the inhibition of GH release (Veber et al., 1981).

Recently, the first somatostatin analogue with antagonist activity has been described (figure 1.5.2e, Fries et al., 1982). Injection of this peptide into the rat completely abolished the inhibitory effects of exogenous SS14 on GH, insulin and glucagon release. In rats, plasma insulin, GH and glucagon levels were significantly increased after treatment with the analogue. This analogue and others developed from it, will be of key importance in



Figure 1.5.2     Structures of Somatostatin 14 and of  
biologically active analogues

a) Structure of somatostatin 14   b) two representations of a prototype structure from which several analogues of potential clinical importance have been developed (From Veber et al., 1981)   c) SMS 201-995 a long acting analogue with a selective action on GH secretion (Bauer et al., 1982)   d) A potent hexapeptide (Veber et al., 1982) and   e) a cyclic pentapeptide with antagonist activity (Fries et al., 1982).



elucidating the role of SS14 and SS28 in the control of physiological processes.

## 1.6 DEGRADATION

Unlike amine and amino acid transmitters, whose duration of action in the nervous system is controlled at least in part by reuptake, the actions of neuropeptides are thought to be terminated exclusively by enzymatic cleavage into inactive fragments. At present, knowledge of the degradation of SS14 and SS28 in brain is very sparse.

[D-Trp<sup>8</sup>] analogues of SS14 display a prolonged duration of action and cleavage of the Trp<sup>8</sup>-Lys<sup>9</sup> bond may be a determining factor in the duration of action of SS14 and SS28. Cathepsin D (EC 3.4.3.23), an acid protease of about 40,000 mol. wt. may be responsible for the degradation of somatostatin in brain (Benuck et al., 1977). This enzyme cleaves at the Trp<sup>8</sup>-Lys<sup>9</sup> bond and also at the Phe<sup>6</sup>-Phe<sup>7</sup> bond. Although abundant in nervous tissue, the enzyme appears to be located predominantly in lysosomes, rather than in the synaptic cleft. Many somatostatin-rich areas of brain contain Cathepsin D but there are some areas (e.g. dorsal horn of spinal cord) where somatostatin nerve terminals are abundant but the enzyme is absent. Other enzymes may, therefore, participate in the degradation of SS14 in the brain. The fate of SS14 and SS28 in the circulation is unknown. An early step in the metabolism of SS14 may be the removal of the N-terminal alanine residue but the [des-Ala<sup>1</sup>] derivative formed retains biological activity (Marki et al., 1981).

## 1.7 CONCLUSIONS

It is now clear that the neural and endocrine functions of somatostatin are more widespread than any other neuropeptide. SSLI exists in tissues not only as the tetradecapeptide but in a number of larger, N-terminally extended forms which may possess functions and targets of action distinct from those of SS14. At present, the pathway by which multiple molecular forms of SSLI are synthesised has not been elucidated.

The rest of this thesis will report the results of studies on the multiple forms of SSLI present in rat and human brain. A sensitive radioimmunoassay and a chromatography system capable of resolving multiple forms of SSLI will be described (Chapter 2). Differences in the multiple forms of SSLI present in the rat hypothalamus and median eminence will be reported (Chapter 3) and these results confirmed by in vitro release studies (Chapter 4). The multiple forms of SSLI present in human post-mortem brain in control cases and in Alzheimer's type dementia and Down's syndrome will be compared (Chapter 5). Finally, the results of a study in which the multiple forms of SSLI in rat hippocampus have been examined after cholinergic denervation of the structure will be described. The implications of this finding in relation to Alzheimer's type dementia will be discussed (Chapter 6).

## CHAPTER 2

### GENERAL METHODS

## 2.1 RADIOIMMUNOASSAY FOR SOMATOSTATIN

### 2.1.1 Introduction

Techniques for the measurement of the physiological concentrations of many biologically active substances require accurate and precise quantitation down to femtomole levels. The technique of radioimmunoassay (RIA), first described for insulin by Yalow & Berson (1960), has proved one of the most satisfactory methods of measuring such low concentrations in biological fluids and tissue extracts.

In radioimmunoassay, a fixed concentration of radioactively labelled antigen is incubated with a constant dilution of antiserum where the number of antigen binding sites is limiting: for example, only 40-50% of the labelled antigen may be bound by the antibody. If unlabelled antigen is added to this system, there is competition between labelled and unlabelled antigen for the limited and constant number of binding sites available. The amount of labelled antigen bound to the antiserum will decrease as the concentration of unlabelled antigen increases. After equilibration, free labelled antigen is separated from antibody-bound labelled antigen and the radioactivity in the bound or free fraction can be counted. In most radioimmunoassays, a second antibody is used to achieve this separation but a number of other methods, such as charcoal or alcohol precipitation, have been described. A calibration or standard curve is set up with increasing concentrations of standard unlabelled antigen and by comparing the displacement of labelled antigen to known concentrations of unlabelled antigen, the concentration of antigen in unknown samples can be calculated.

Tubes containing labelled antigen only (total counts: TC), are included with the standard curve. Non-specific binding of labelled antigen in the absence of antiserum is the blank value (Bk) and is expressed as a percentage of total counts added. The maximal binding of the labelled antigen at any given antiserum concentration (Bo), is the percentage which binds to the antiserum in the absence of unlabelled antigen.

The first radioimmunoassay for somatostatin (Arimura et al., 1975) was described soon after the isolation and purification of the peptide from hypothalamus and subsequently a number of other workers have reported sensitive and specific radioimmunoassays for somatostatin (Kronheim et al., 1976; Patel & Reichlin, 1978; Penman et al., 1979). In addition, a number of kits are commercially available.

#### 2.1.2 Preparation of somatostatin antiserum

Antiserum is prepared by injecting the antigen, usually in an oil/water emulsion containing an adjuvant into a suitable animal. This is usually a rabbit, although guinea pigs, sheep or donkeys are sometimes used. Proteins and large peptides are immunogenic in their own right but small peptides such as SS14 and other small molecules have to be coupled to a carrier protein in order to elicit an immune response. The coupling of different parts of the peptide to the carrier protein can be used to give antisera of differing specificity. The somatostatin antiserum used in this radioimmunoassay was raised by Dr. A.J. Harmar and Dr. P. Keen in Bristol by a method based on that described for neurotensin (Carraway & Leeman, 1976). In this case, succinylated

thyroglobulin was used as carrier coupled to the N-terminus of the SS14 molecule giving a C-terminal specific antiserum.

40mg bovine thyroglobulin (Sigma Chemical Co., Poole, Dorset) were dissolved in 8ml 0.15M NaCl, and the pH adjusted to 7 with  $\text{Na}_2\text{CO}_3$ . 80mg solid succinic anhydride were added over 1 hour, the pH being kept close to 7 by constant adjustment with  $\text{Na}_2\text{CO}_3$ . After a further 30 min the product was dialyzed against distilled water and lyophilized. 20mg succinylated thyroglobulin were dissolved in 1ml of 0.1M phosphate buffer pH 7.4 and 10mg SS14 (UCB Bioproducts, Brussels, Belgium) was added. Conjugation of SS14 to succinyl thyroglobin was achieved by the dropwise addition of 10mg 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (Sigma:  $20\text{mg ml}^{-1}$  in phosphate buffer pH 7.4). The reaction mixture was stirred in the dark for 20 hours, dialyzed for 48 hours against distilled water (5 litres) and lyophilized. Half of the resulting conjugate was suspended in 4.5ml 0.15M NaCl and emulsified with an equal volume of Freund's complete adjuvant supplemented with heat-killed tubercle bacilli ( $5\text{mg ml}^{-1}$ ; Difco, Detroit, MI). 2ml of the immunogen was administered by multiple intradermal injections to each of four New Zealand white rabbits together with a single subcutaneous injection of pertussis vaccine (Difco, 0.5ml). Six weeks later the animals were boosted with a further 2ml of the immunogen and bled from the ear vein at 10-14 day intervals. The antiserum used for radioimmunoassay was obtained from a single rabbit seven weeks after the booster dose of immunogen was administered.



### 2.1.3 Iodination

#### i) Introduction

A variety of methods are available for the radioiodination of peptides and proteins. Protein iodination methods can be broadly divided into two groups, direct methods in which radioiodine is incorporated directly into tyrosyl or histidyl residues of the protein chain and conjugation methods in which an iodinated moiety is conjugated to a specific sidechain of the protein. Most conjugation methods involve the modification of  $\text{NH}_2$  sidechains and N-terminal amino groups.

Chloramine T is the most widely used reagent for the direct iodination of peptides and proteins (Hunter & Greenwood, 1962). However, other direct methods have been described; the use of insoluble oxidising agents such as Iodogen (Fraker & Speck, 1978) and enzymatic iodination using lactoperoxidase and hydrogen peroxide (Marchalonis, 1969). N-succinimidyl 3-(4-hydroxy 5-[ $^{125}\text{I}$ ]iodophenyl) propionate (Bolton and Hunter reagent) is the most commonly used conjugation labelling reagent (Bolton & Hunter, 1973) although others such as p-hydroxybenzimidate (Wood et al., 1975) have been described.

Methods using both lactoperoxidase and chloramine T for the iodination of SS14 have been described (Penman et al., 1979; Rorstad et al., 1979; Patel & Reichlin, 1978). In one study (Penman et al., 1979) a comparison of labelled antigen generated by both methods and used under identical conditions showed that the label behaved identically with respect to stability and antibody binding when purified on octadecyl silica. One report has

described the use of Iodogen for the radioiodination of SS14 (Conlon et al., 1981) to produce labelled antigen of high specific activity.

In this study, all three direct methods of radioiodination were evaluated to establish which method would provide labelled antigen with optimum sensitivity.

The sequence of SS14 does not contain either a tyrosyl or histidyl residue and therefore a modified sequence must be used for iodination. Three peptides are commonly used: Tyr<sup>1</sup>-SS14, in which the N-terminal alanyl residue is replaced by tyrosine, Tyr<sup>11</sup>-SS14, where Phe<sup>11</sup> is replaced by tyrosine and the fifteen amino acid peptide Tyr<sup>0</sup>-SS14, which has a tyrosyl residue added at the N-terminus of the molecule.

Both <sup>125</sup>I-Tyr<sup>1</sup>-SS14 and <sup>125</sup>I-Tyr<sup>0</sup>-SS14 gave similar standard curves in a number of systems (Arimura et al., 1978; Patel & Reichlin, 1979), which differed considerably from the curve for <sup>125</sup>I-Tyr<sup>11</sup>-SS14. <sup>125</sup>I-Tyr<sup>0</sup>-SS14 can be stored for 5-6 times longer than <sup>125</sup>I-Tyr<sup>1</sup>-SS14 without a deterioration in assay performance (Arimura et al., 1978) and so was used as the substrate for iodination in this study.

#### ii) Chloramine T Method

Na <sup>125</sup>I is oxidised by chloramine T in the presence of the peptide or protein to be labelled with the subsequent incorporation of radioiodide into tyrosyl residue(s) (Hunter & Greenwood, 1962). Excess chloramine T can be reduced by the addition of a reagent such as sodium metabisulphite.

Tyr <sup>0</sup> -SS14	1mg ml <sup>-1</sup> in 20mM Acetic acid
Chloramine T	0.5mg ml <sup>-1</sup> in 0.5M Phosphate buffer pH 7.4
Na <sup>125</sup> I	Amersham IMS 30: 3.7GBq ml <sup>-1</sup>
Bovine serum albumin (BSA)	0.1mg ml <sup>-1</sup> in 0.2% (v:v) trifluoroacetic acid (TFA)
TFA/KI	10mg ml <sup>-1</sup> KI in 0.2% (v:v) TFA

10 $\mu$ l (37MBq) Na<sup>125</sup>I was placed in a polystyrene reaction vessel. 20 $\mu$ l chloramine T solution and 2 $\mu$ g Tyr<sup>0</sup>-SS14 (20 $\mu$ l of a 1:10 dilution of the stock solution) were added. After 30 sec the reaction was stopped by the addition of 100 $\mu$ l of the BSA solution followed by 700 $\mu$ l of TFA/KI. Sodium metabisulphite was not used to stop the reaction in order to avoid the reduction of the disulphide bond in SS14.

## ii) Lactoperoxidase method

A more gentle method for radioiodination using the enzyme lactoperoxidase to catalyse the oxidation of iodide in the presence of very small amounts of hydrogen peroxide, was first described for the iodination of immunoglobulins (Marchalonis, 1969). The method is now widely used for the preparation of labelled antigens for radioimmunoassay.

Tyr <sup>0</sup> -SS14	1mg ml <sup>-1</sup> in 20mM Acetic acid
lactoperoxidase	0.5mg ml <sup>-1</sup> in 0.1M sodium Acetate pH 5.6
Na <sup>125</sup> I	Amersham IMS 30: 3.7 GBq ml <sup>-1</sup>
Sodium acetate	0.4M pH 5.6
H <sub>2</sub> O <sub>2</sub>	0.03% solution
TFA/KI	10mg ml <sup>-1</sup> KI in 0.2% (v:v) TFA

10 $\mu$ l (37MBq) Na<sup>125</sup>I was placed in a polystyrene reaction vessel.

10 $\mu$ l lactoperoxidase solution, 25 $\mu$ l 0.4M sodium acetate and 1 $\mu$ g Tyr<sup>0</sup>-SS14 were added. The reaction was initiated by the addition of 5 $\mu$ l of H<sub>2</sub>O<sub>2</sub> solution. After 75 sec the reaction was stopped by dilution by the addition of 800 $\mu$ l TFA/KI solution.

iv) Iodogen method

To avoid the harmful effects of soluble oxidising agents such as chloramine T, the use of 1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycoluril (Iodogen) has been reported (Fraker & Speck, 1978). Iodogen is coated onto the surface of the reaction vessel and iodination effected by the addition of Na<sup>125</sup>I and peptide solution.

Tyr <sup>0</sup> -SS14	0.5mg ml <sup>-1</sup> in 0.0 5M phosphate buffer pH 7.4
Iodogen	40 $\mu$ g ml <sup>-1</sup> in dichloromethane
Na <sup>125</sup> I	Amersham IMS 30: 3.7 GBq ml <sup>-1</sup>
TFA/KI	10mg ml <sup>-1</sup> in 3% (v:v) TFA

50 $\mu$ l Iodogen solution was evaporated to dryness under nitrogen in a polypropylene reaction vessel. 5 $\mu$ g Tyr<sup>0</sup>-SS14 (10 $\mu$ l of stock solution) and 10 $\mu$ l (37MBq) Na<sup>125</sup>I were added and mixed together. After a 10 min incubation the iodination was interrupted by the addition of 200 $\mu$ l 0.05M phosphate buffer and 500 $\mu$ l TFA/KI solution, followed by removal from the oxidising environment.

v) Purification of <sup>125</sup>I-Tyr<sup>0</sup>-SS14 by Liquid Chromatography

The aim of purification is to isolate the iodinated peptide or protein from the iodination reaction mixture. Ideally the iodinated peptide should not only be separated from unreacted iodide but also from unreacted peptide and from multiply iodinated forms of

the peptide. Generally, gel filtration chromatography is used but a number of other methods including ion-exchange chromatography, affinity chromatography and high performance liquid chromatography have been described.

High performance liquid chromatography (HPLC) is particularly suitable for the purification of labelled antigen because it is able to separate monoiodinated material from diiodinated and unlabelled material, as well as removing unreacted iodide.

Methods describing the purification of iodinated SS14 by ion-exchange chromatography (Arimura et al., 1975), Sephadex G-25 gel filtration chromatography (Kronheim et al., 1976; Patel & Reichlin, 1978) and octadecyl ( $C_{18}$ ) silica liquid chromatography (Penman et al., 1979) have been published.

In this study  $^{125}\text{I}$ -labelled Tyr<sup>0</sup>-SS14 was purified from the reaction mixture by HPLC (Harmar & Rosie, 1984). The chromatography system consisted of a two-channel minipump (Milton Roy Co., Riviera Beach, FL) connected in series to a sample injection valve with a 1ml loop (Rheodyne Berkeley, CA) and a guard column holder (Brownlee MPLC cartridge system: Brownlee Labs. Santa Clara, CA). Usually a 3cm x 0.46 cm disposable column of cyanopropyl silica (Spheri 5 cyano: Brownlee Labs) was used although in some cases a similar sized column of  $C_{18}$  silica (RP-18 Spheri 5: Brownlee Labs) was used with comparable results. The two channels of the pump were primed respectively with 0.2% aqueous TFA (Solvent A) and 0.2% TFA in methanol (Solvent B). After equilibration of the column in Solvent A, the iodination mixture was injected and eluted with a stepwise gradient of Solvent B from 0%-100% in increments of 10%. The flow rate was  $1\text{ml min}^{-1}$ .

and six fractions of 1ml were collected at each step in the gradient. The elution profile of  $^{125}\text{I}$ -labelled Tyr<sup>0</sup>-SS14 from an iodination Iodogen using is shown (figure 2.1.1). Three peaks of radioactivity were obtained. The first peak (peak I) consisted of free  $^{125}\text{I}$  which passed unretarded through the column. Two further peaks of radioactivity were generally observed (peaks II & III) eluting at 30% and 40% methanol respectively. These two peaks may correspond to mono-iodinated, and di-iodinated forms of  $^{125}\text{I}$ -labelled Tyr<sup>0</sup>-SS14. Although both labels exhibited high antibody binding and produced similar standard curves (figure 2.1.2), material in peak II was routinely used for radioimmunoassay.

vi) Comparison of methods

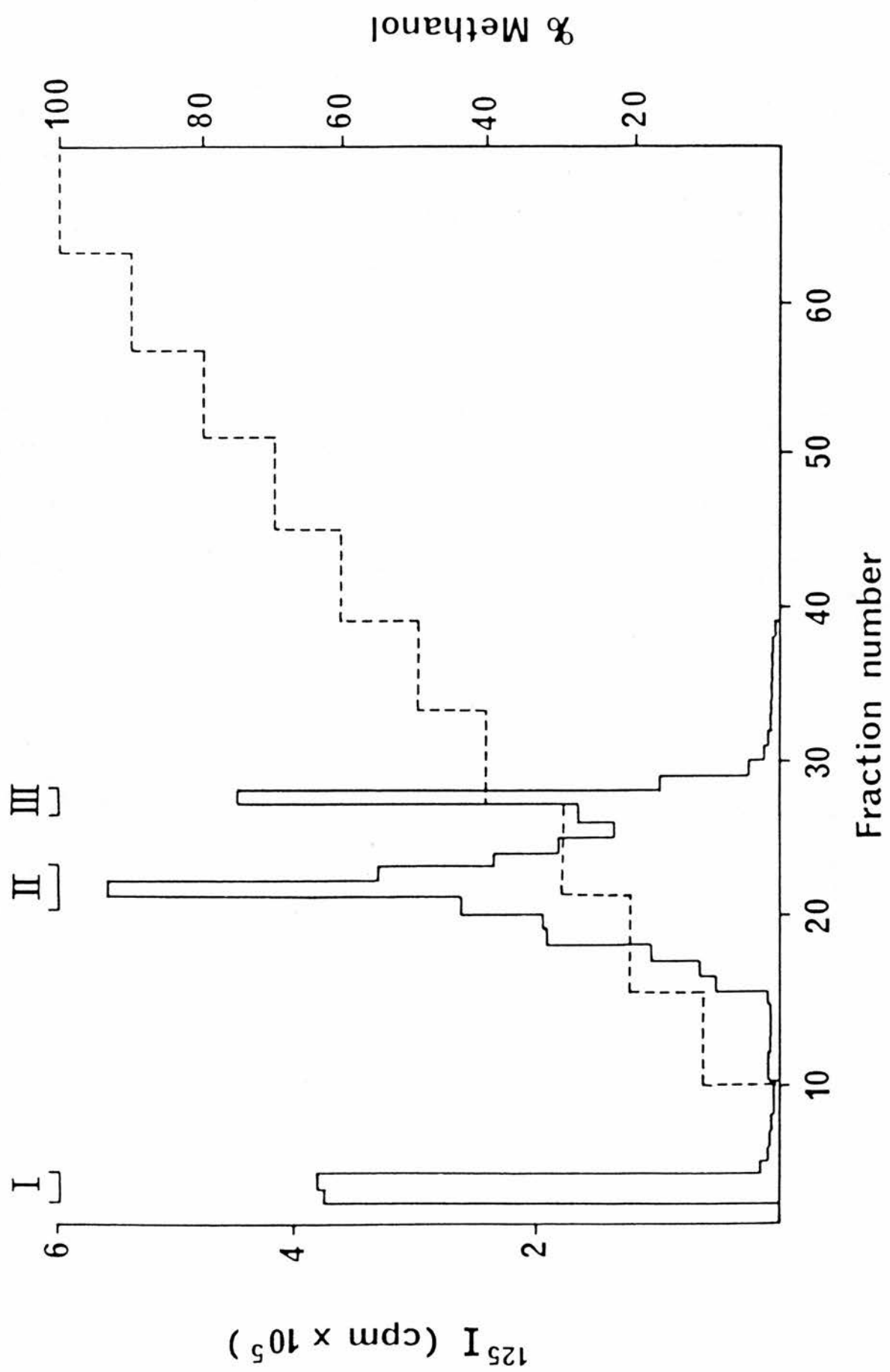
In the study of Penman et al. (1979) somatostatin label produced by lactoperoxidase iodination and purified on CM cellulose was superior to labelled antigen generated by chloramine T. The labelled antigens which gave highest binding were produced when C<sub>18</sub> silica liquid chromatography was used for purification, irrespective of whether chloramine T or lactoperoxidase was used as iodinating agent. For this reason, only HPLC was used to purify  $^{125}\text{I}$ -labelled Tyr<sup>0</sup>-SS14.

Salacinski et al. (1981) had shown that for the majority of proteins and peptides tested, Iodogen gave higher iodine incorporation with less iodination damage than both chloramine T and lactoperoxidase. In addition, Conlon et al. (1981) had shown that Tyr<sup>11</sup>-SS14 could be successfully iodinated using Iodogen.

In this study, using either cyanopropyl silica liquid chromatography or C<sub>18</sub> silica liquid chromatography to purify the

Figure 2.1.1     Isolation of  $^{125}\text{I}$ -labelled Tyr<sup>0</sup>-SS14 by liquid chromatography

Tyr<sup>0</sup>-SS14 was iodinated and the reaction products were separated by liquid chromatography as described in section 2.1.3. The solid line shows c.p.m. per fraction and the broken line per cent methanol in eluant. The significance of peaks I, II and III is discussed in the text.





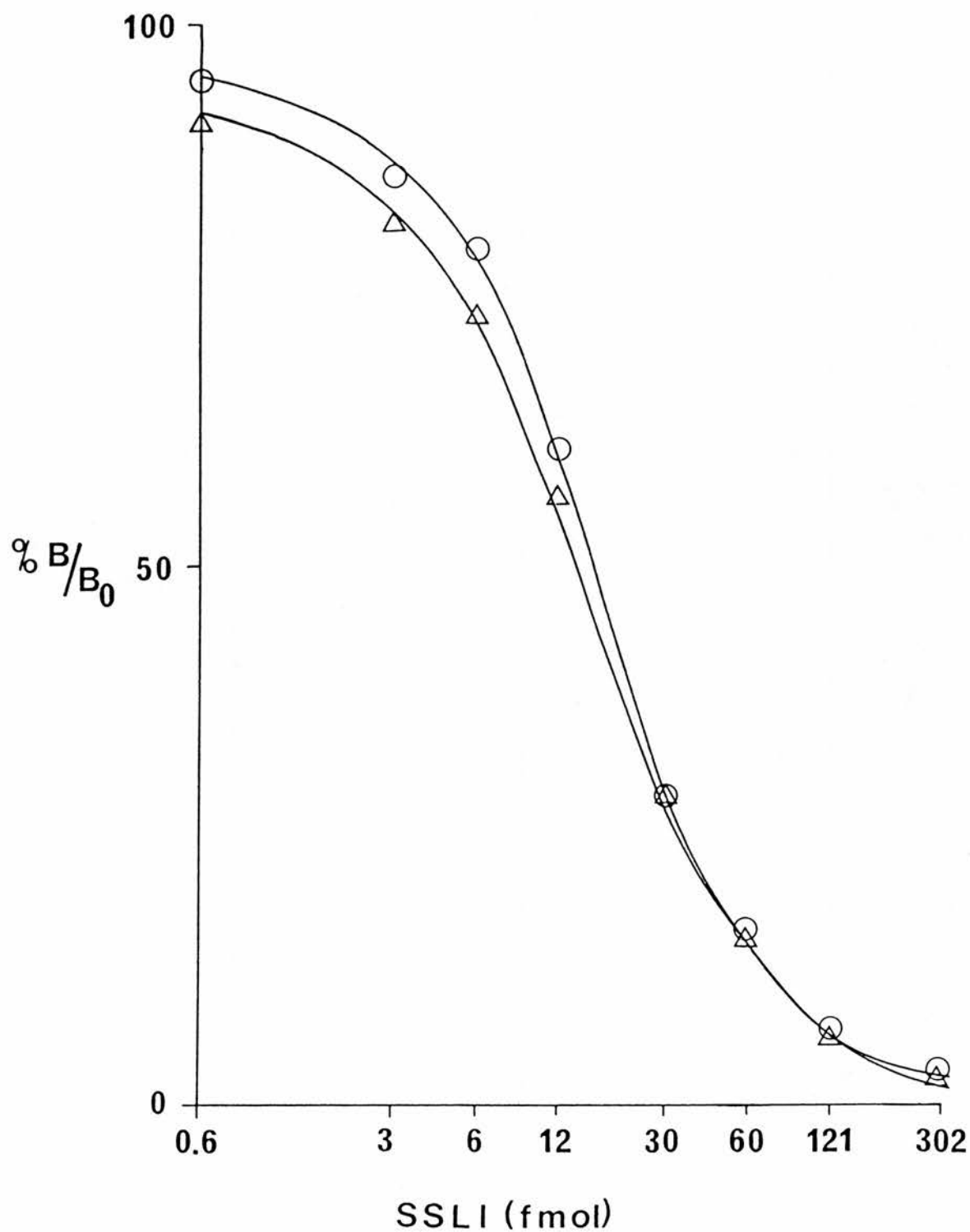


Figure 2.1.2 Comparison of binding of peaks II and III from iodination using Iodogen

Tyr<sup>0</sup>-SSL14 was iodinated using Iodogen as described in section 2.1.3. Displacement curves using material from peaks II and III were similar.

○ — material from peak II  
 △ — material from peak III

labelled peptide, the lactoperoxidase method gave similar or slightly better results than that of chloramine T. The success of the lactoperoxidase method varied between individual enzyme preparations and although occasionally producing labelled antigen which exhibited high antibody binding was unreliable. Similarly, in this study, chloramine T proved unreliable.

Iodination using Iodogen produced a labelled antigen which increased the sensitivity of the RIA by 5-6 fold compared to iodination using lactoperoxidase (figure 2.1.3).

Salacinski et al. (1981) had found that the storage of radiolabelled antigen after the addition of methanol preserved immunogenicity better than other storage methods including freeze-drying. The length of time that a labelled antigen could be stored and used before a significant alteration in the sensitivity of the RIA was observed, was increased when an equal volume of methanol containing 0.2% TFA was added to the labelled antigen. This time was also significantly increased when label was stored at  $-40^{\circ}\text{C}$  containing methanol/0.2% TFA compared to storage at  $-70^{\circ}\text{C}$  with or without the addition of methanol. This suggests that the immunogenicity of somatostatin label is best preserved at low temperature but without freezing. Under optimal storage conditions  $^{125}\text{I-Tyr}^0\text{-SS14}$  produced by lactoperoxidase iodination was useable for about six weeks before a significant reduction in the value of  $B_0$  was observed. Label produced using Iodogen could be used for up to three months without a significant deterioration in the performance of the radioimmunoassay.

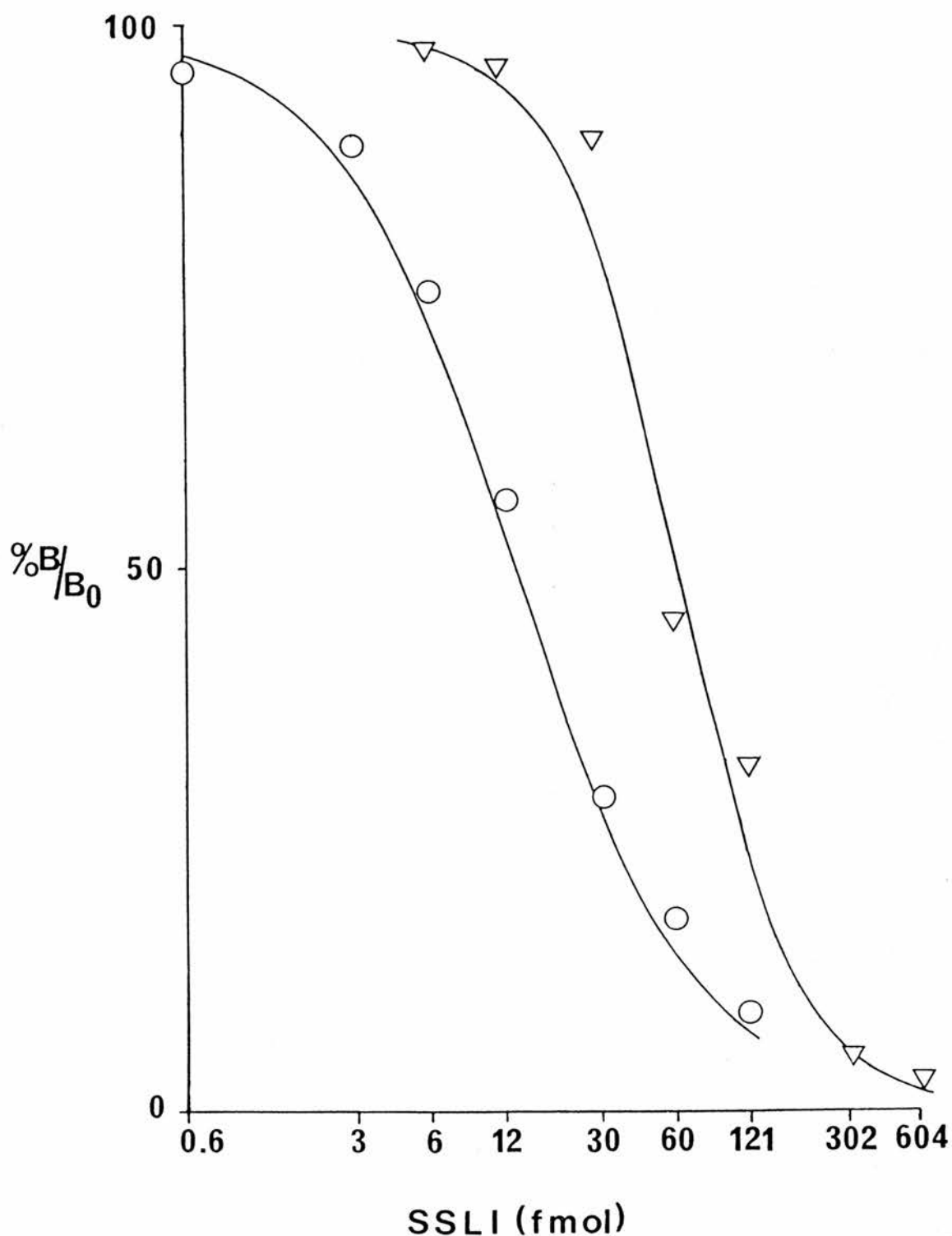


Figure 2.1.3 Comparison of binding of  $^{125}\text{I}$ -Tyr<sup>0</sup> produced by iodination using lactoperoxidase or Iodogen

Tyr<sup>0</sup>-SS14 was iodinated using lactoperoxidase or Iodogen as described in section 2.1.3. Displacement curves of standard SS14 showed that the Iodogen method produced labelled antigen which increased the sensitivity of the RIA by 5-6 fold compared to material iodinated using lactoperoxidase

- — Iodogen
- ▽ — lactoperoxidase

#### 2.1.4 Optimisation of Assay Conditions

In order to determine a suitable antiserum dilution to give maximum sensitivity, an antiserum dilution curve was set up (figure 2.1.4) with dilutions ranging from 1:2,500 to 1:1,000,000. In addition a series of standards of SS14 were assayed at each dilution (figure 2.1.5) to show changes in sensitivity as antiserum concentration was reduced. An antiserum dilution of 1:250,000 in assay buffer (1:750,000 final dilution) gave a  $B_0$  value of 40-50% and was used in all further studies.

Most reported assays for somatostatin operate at pH 7.0-7.8 and in the study of Penman et al. (1979) optimal sensitivity was obtained at pH 7.4. In order to confirm this finding, assays were performed at pH 6 and pH 7.6 using phosphate buffer and at pH 8.6 in barbital buffer. Although the results obtained at pH 6 and pH 7.6 were similar, the blank was 3-4 times higher and  $B_0$  was reduced by approximately 50% at pH 8.6. Assay buffers at pH 7.6 containing 0.1% and 0.5% BSA were compared and gave comparable blanks and maximum binding. Detergent (0.1% (v:v) Tween 20: BDH) improved binding at pH 8.6 but not at pH 6 or pH 7.6. In common with other radioimmunoassays for somatostatin, pH 7.6 was selected.

The use of Trasylol (aprotinin: Bayer U.K.) has been recorded in a number of published radioimmunoassays for somatostatin (Patel & Reichlin 1978; Penman et al., 1979) in amounts ranging from 2,000-5,000 KIU ml<sup>-1</sup>. EDTA 0.025-0.05M is also generally added. The inclusion of Trasylol + EDTA in the buffer should help to protect the sample and tracer from enzymatic degradation.

Trasylol (2500 KIU ml<sup>-1</sup>) and EDTA (0.5M) in the antiserum buffer resulted in increased  $B_0$  and reduced blank values. The

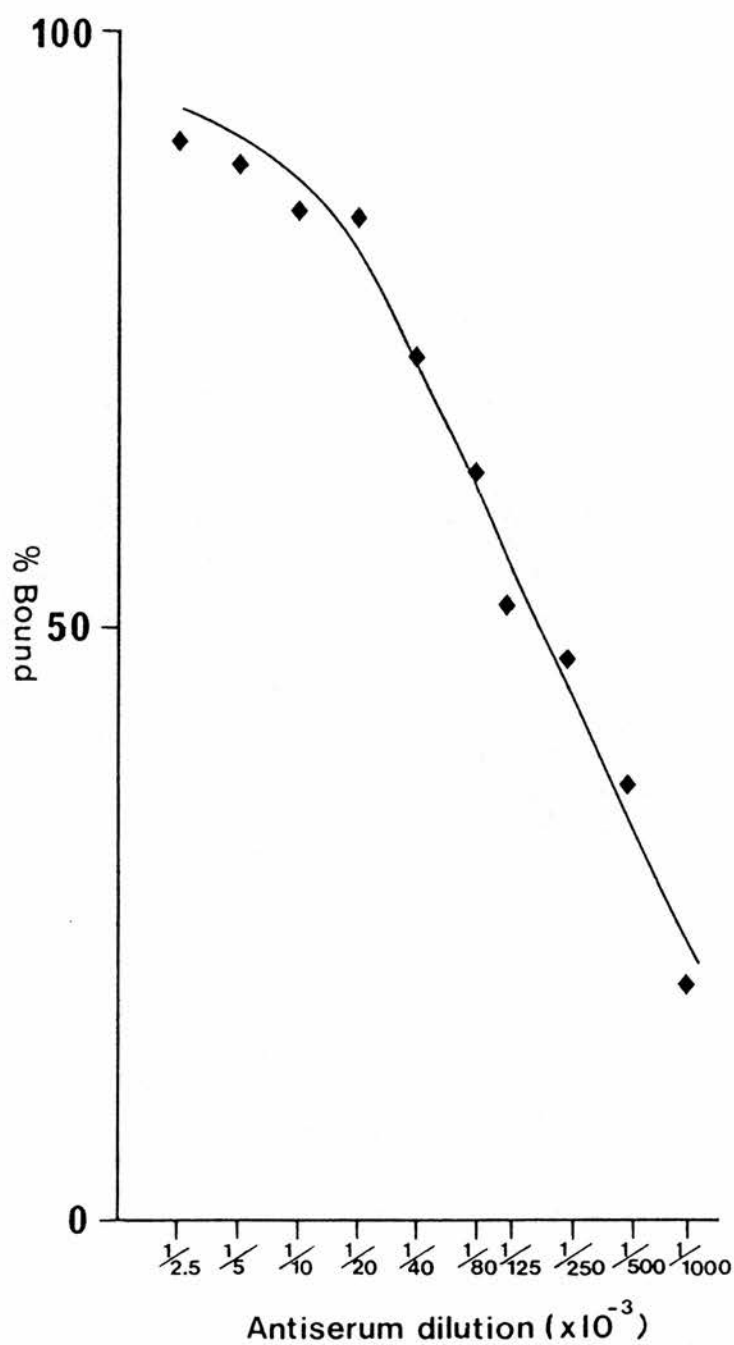


Figure 2.1.4 Binding of  $^{125}\text{I}$ -Tyr<sup>0</sup>-SS14 at increasing antiserum dilution

$^{125}\text{I}$ -labelled-Tyr<sup>0</sup>-SS14 binding was reduced as the antiserum dilution was increased. 40-50% binding was obtained at a dilution of 1:250,000

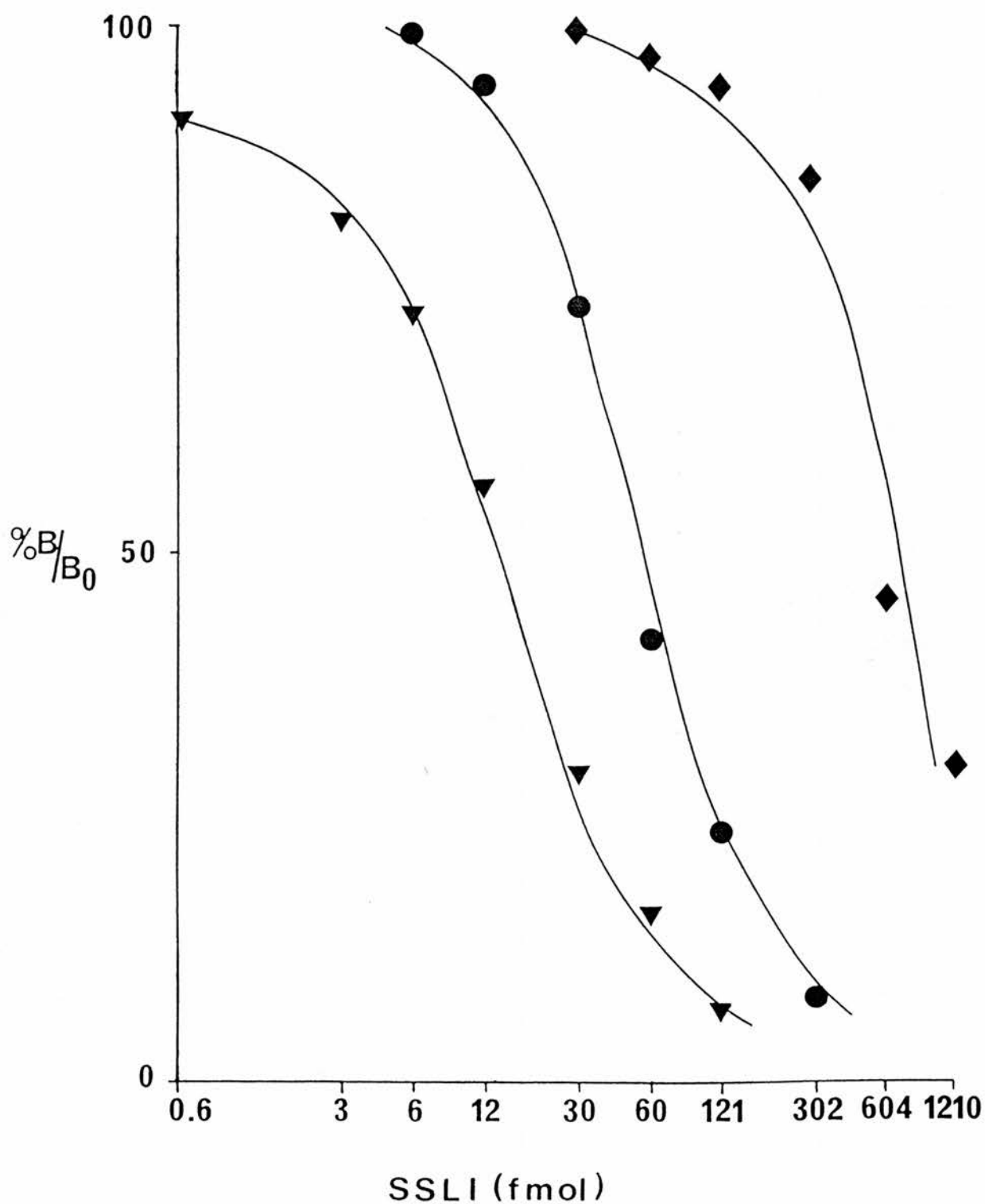


Figure 2.1.5 Standard curves for SSLI at differing antiserum dilution

Standard curves were set up with standards of SS14 ranging from 0.6 to 1210fmol in 200 $\mu$ l. Antiserum was used at three dilutions.

- ◆ - 1:20,000
- - 1:80,000
- ▼ - 1:250,000

sensitivity of the assay was also improved.

In most radioimmunoassays, the use of a second antibody is the method of choice for the separation of label bound to antiserum from free label. However, methods employing both a second antibody (Patel & Reichlin, 1978; Penman et al., 1979) dextran- or BSA-coated charcoal (Kronheim et al., 1976; Arimura et al., 1978) and propanol precipitation (Morel et al., 1981), have been published for the separation of bound  $^{125}\text{I}$ -labelled somatostatin from free label.

In this study, a second antibody method and dextran- and BSA-coated charcoal methods were evaluated for their suitability in RIA. Using standard second antibody techniques, an average blank value of  $17.5 \pm 2.8\%$  (mean  $\pm$  S.D.) over seven assays was obtained with an average  $B_0$  of  $39.7 \pm 9.6\%$  (mean  $\pm$  S.D.). This high blank may be due to a somatostatin binding protein present in the precipitating serum (Ogawa et al., 1977). All charcoal methods gave significantly lower blanks whilst improving maximum binding at the same antiserum concentration. A comparison of three charcoal types:

- i) Sigma C 4386 activated washed with HCl (Sigma)
- ii) Norit PN5 activated (BDH)
- iii) Norit GSX low in chloride (BDH)

showed that the highest binding and lowest blank value under identical conditions was obtained using Sigma C4386. The use of different concentrations of this charcoal, BSA and the substitution of dextran T70 (Pharmacia) for BSA showed that a  $5\text{mg ml}^{-1}$  charcoal suspension in 0.1M phosphate buffer pH 7.6 containing  $2\text{mg ml}^{-1}$  BSA gave the lowest blank value with similar binding compared with other preparations.

In order to increase the speed of addition of the charcoal suspension, 800 $\mu$ l of phosphate buffer pH 7.6 was added followed by 200 $\mu$ l of a 5x charcoal concentrate (25mg ml<sup>-1</sup> charcoal suspension in phosphate pH 7.6 containing 10mg ml<sup>-1</sup> BSA) using an Oxford Microdoser pipette (BCL, Lewes, East Sussex).

Charcoal suspension was added at 4°C and tubes were incubated on ice for 20 min before centrifugation. Comparable blanks and maximal binding values were obtained with incubation times of between 20 and 40 minutes after the addition of charcoal suspension with blank values increasing after this time. Since it takes 5-10 min to add charcoal to 288 tubes (the maximum number of tubes in one centrifuge run) 20 min was selected as the incubation time after charcoal had been added to the final tube.

Optimal conditions for this radioimmunoassay were obtained using the following reagents:

- |                          |  |
|--------------------------|--|
| i) Assay buffer          | 0.1M phosphate buffer pH 7.6 +<br>1mg ml <sup>-1</sup> BSA                     |
| ii) Antiserum buffer     | assay buffer containing 2500 KIU ml <sup>-1</sup><br>Trasyolol + 0.05M EDTA    |
| iii) Charcoal Suspension | 5mg ml <sup>-1</sup> in phosphate buffer pH 7.6 +<br>2mg ml <sup>-1</sup> BSA. |

#### 2.1.5 Operation of assay

##### i) Sample preparation

Samples for the radioimmunoassay of somatostatin were usually extracted from tissues by homogenisation in acetic acid or obtained by chromatography in buffer systems containing methanol or acetonitrile. Since all of these solvents are unsuitable for use in RIA, samples were evaporated to dryness in a vacuum oven at room



temperature before RIA. Displacement curves using serial dilutions of rat and human brain prepared in this way exhibited parallelism with standard curves for SS14 (figure 2.1.6).

ii) Assay procedure

Incubation tubes contained the following:

Sample	200 $\mu$ l in assay buffer
$^{125}$ I-labelled Tyr <sup>0</sup> -SS14	200 $\mu$ l (5000 cpm) in assay buffer
Antiserum	200 $\mu$ l (1:250,000 dilution) in antiserum buffer

In each assay, a standard curve consisting of duplicate samples containing 0.6, 3.0, 6.0, 12.0, 30.2, 60.4 and 120.8fmol of synthetic SS14 (Cambridge Research Biochemicals, Harston, Cambridgeshire) in 200 $\mu$ l, were included. Tubes for the determination of total counts, blank and Bo values were also set up in duplicate. After incubation of assay tubes for 20-24 hours at 4°C, 800 $\mu$ l phosphate buffer pH 7.6 was added followed by 200 $\mu$ l concentrated charcoal suspension. The tubes were vortexed and incubated on ice for 20 min prior to centrifugation (1,600g for 30 min at 4°C). After decantation, activity in the supernatant (which contains bound tracer) was counted for four minutes using a Berthold MAG 310 or MAG 315 gamma counter. Standard curves were plotted and unknowns estimated by the counter using standard computer programmes.

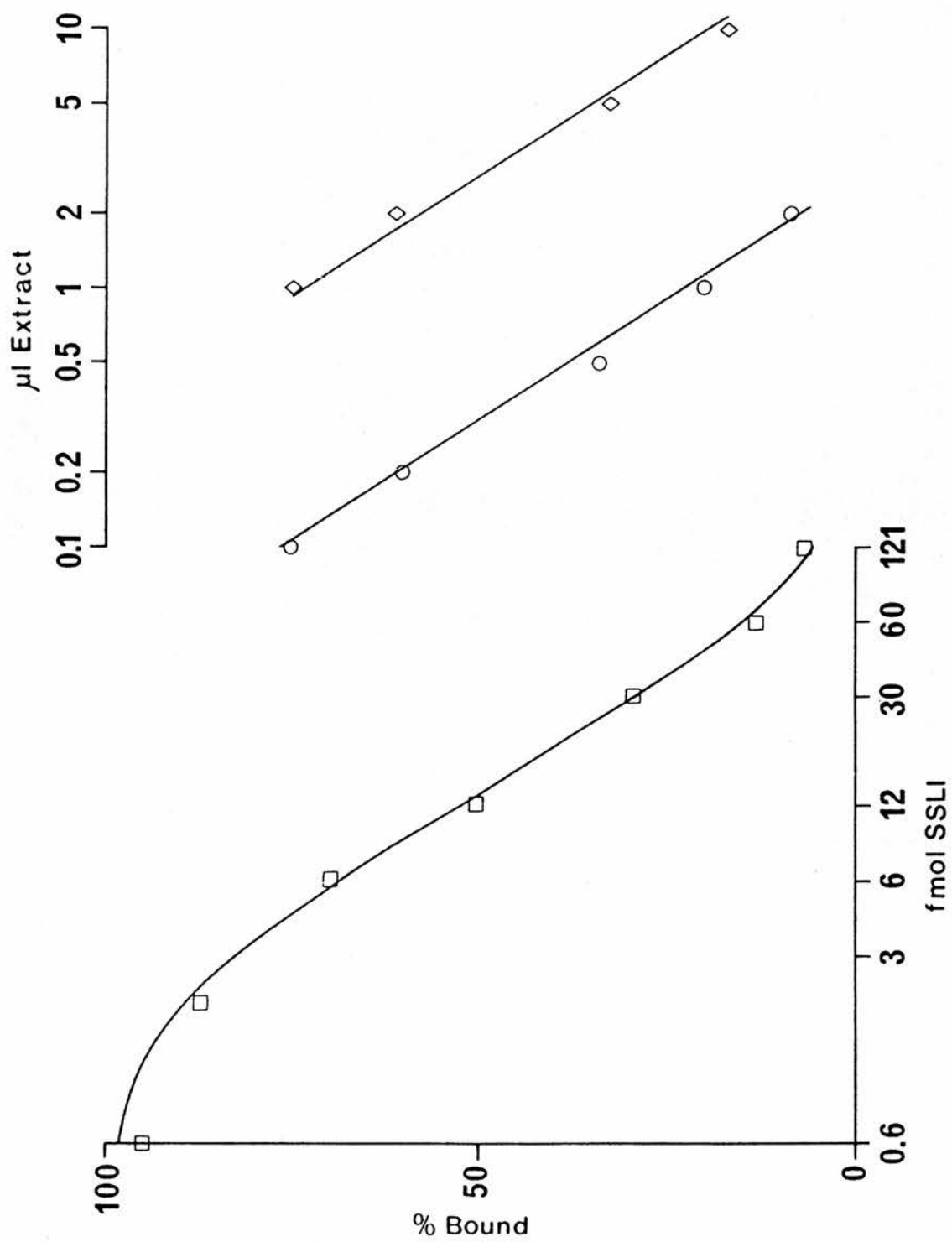
iii) Quality control

Pools for the determination of inter- and intra-assay co-efficients of variation, prepared from an acetic acid extract of rat brain, were included in all assays. Pools were dried in a

Figure 2.1.6      Comparison of displacement curves produced by synthetic SS14 and serial dilutions of tissue extracts

Extracts of rat and human brain showed parallelism with standard SS14.

- = SS14
- = rat
- ◇ = human



vacuum oven with the samples prior to radioimmunoassay. The intra-assay coefficient of variation (COV) was 4% (measured at both 20 and 60 fmol:  $n = 10$ ) and the inter-assay COV was 6.5% at 20fmol and 16% at 60fmol. The sensitivity of the assay, defined as the amount of SS14 which reduced binding of  $^{125}\text{I}$ -labelled  $\text{Tyr}^0\text{-SS14}$  to 90% of the binding observed in the absence of added SS14 was  $2.2 \pm 0.85\text{fmol}$  (mean  $\pm$  S.D.,  $n = 13$ ).

iv) Hormonal specificity

Since SS14 is a cyclic peptide containing a disulphide bridge linking cysteine residues at positions 4 and 14, the synthesis of fragments suitable for the investigation of antiserum specificity is difficult. However, the antiserum appeared to be directed against the C-terminal or central portion of the molecule, since SS28 was equipotent to SS14 in displacing labelled SS14 from the antiserum whereas  $\text{SS28}_{(1-14)}$  was not detected in concentrations up to 20ng in 200 $\mu\text{l}$  (figure 2.1.7). High molecular weight forms of somatostatin were also detected by this antiserum. No pure preparations of HMW-SS have been provided for RIA and so it was impossible to establish the exact crossreactivity of these peptides with the antiserum. Serial dilution of HMW-SS exhibited a parallel displacement curve with standard SS14. Purified human prolactin, GH, follicle stimulating hormone (FSH), TSH, ovine luteinizing hormone (LH) (NIADDK, Bethesda MD) synthetic  $\beta$ -endorphin, dynorphin A, corticotropin-releasing hormone (CRH), ACTH, neurotensin and VIP (Peninsula Labs. Belmont CA) were tested to determine antibody specificity. None of these peptides cross-reacted with the antiserum at concentrations up to 20ng in a 200 $\mu\text{l}$  sample.

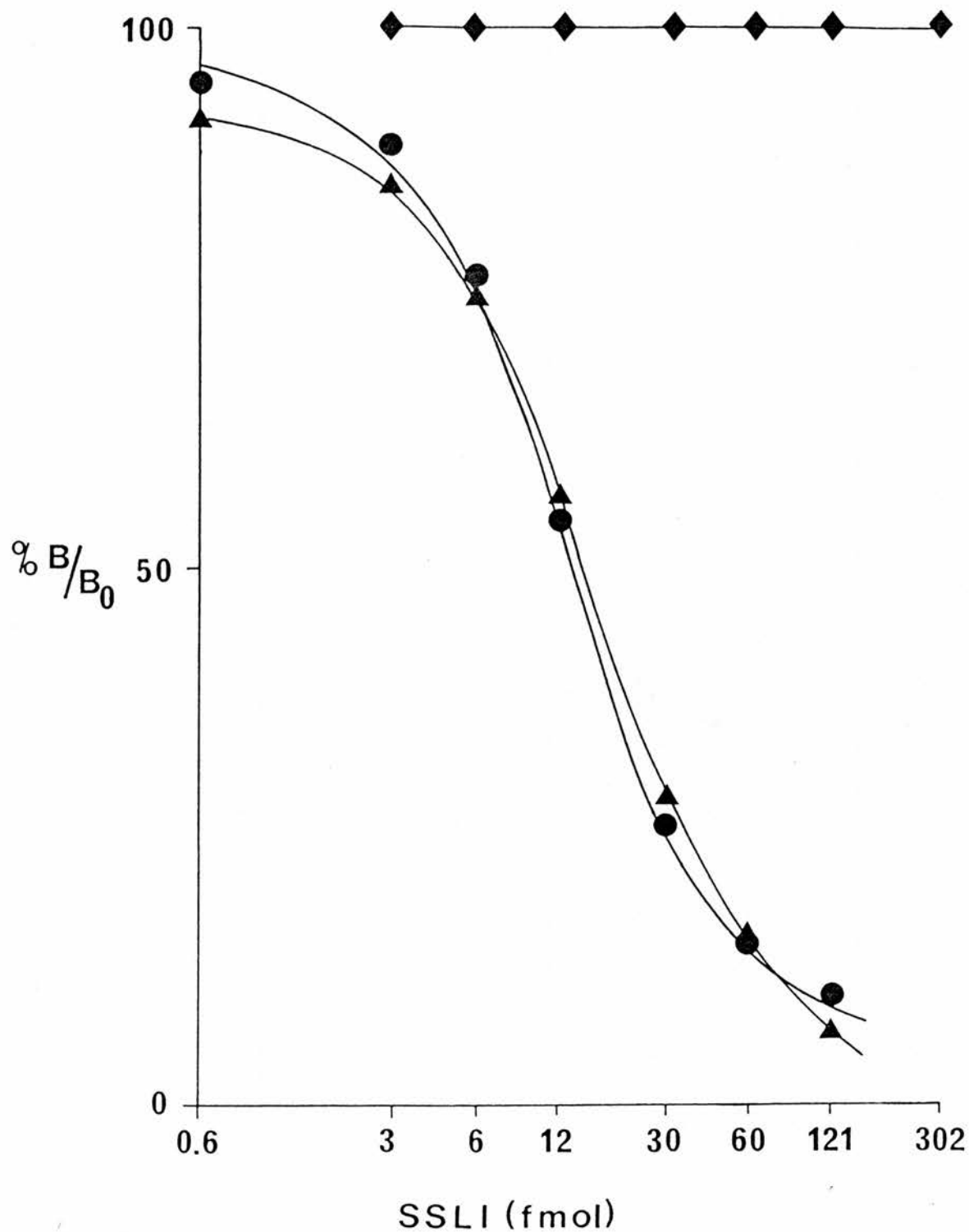


Figure 2.1.7 Crossreactivity of peptides with somatostatin antiserum

Displacement curves for SS28, SS28(1-14) and other peptides are shown. SS28 crossreacted fully with the antiserum whilst SS28(1-14) did not crossreact.

- — SS14
- ▲ — SS28
- ◆ — SS28(1-14), prolactin, GH, FSH, TSH, LH  $\beta$ -endorphin  
Dynorphin A, DynA, CRH, ACTH, Neurotensin, VIP

#### 2.1.6 Comparison with other radioimmunoassays for somatostatin

The assay described here differs from published radioimmunoassays for somatostatin in the method used for the generation of  $^{125}\text{I}$ -labelled SS14. Most published methods use either chloramine T or lactoperoxidase for iodination and gel filtration or ion exchange chromatography is used to separate free  $^{125}\text{I}$ -iodide from radioiodine incorporated into peptide. Iodogen proved to be the most reliable method for the iodination of Tyr<sup>0</sup>-SS14 and a label with a comparable  $B_0$  value and sensitivity was produced after each iodination. This was not observed using either chloramine T or lactoperoxidase.

Octadecyl silica purified material using lactoperoxidase iodination did not significantly alter its binding during two months storage (Penman et al., 1979) and Arimura et al. (1979) report storage of chloramine T iodinated Tyr<sup>0</sup>-SS14 for six to eight weeks. In this study, iodination using Iodogen followed by HPLC using cyanopropyl silica produced a labelled antigen with maximal binding being unchanged after three months when stored in methanol at  $-40^\circ\text{C}$ . Other proteins and peptides produced by the Iodogen method have also been reported to be stable for this period of time (Salcinski et al., 1981).

With a mean sensitivity of 2.2fmol (3.64pg), the assay performed well in comparison with other published assays. Sensitivity in other assays ranged from 2-15pg, although in most cases the method of calculating sensitivity was not given.

Published assays quote intra-assay COV between 5-12% and inter-assay COV ranging from 10-17%. This assay performs as well as, or better than, most with intra-assay COV of only 4% at both 20

and 60fmol and inter-assay COV of 6.5% at 20fmol and 16% at 60fmol.

Most assays use the second antibody method to separate bound from free label. However, the use of BSA coated charcoal and the counting of decanted supernatant has been reported (Patel & Reichlin, 1978). By comparison of inter- and intra-assay COV figures, it can be seen that the use of charcoal in this assay is as reliable as second antibody methods published by others.

## 2.2 SEPARATION OF MULTIPLE FORMS OF SOMATOSTATIN BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

### 2.2.1 Introduction

The techniques of liquid chromatography are based on the interaction between solutes, a liquid mobile phase and a solid stationary phase. Various liquid chromatographic techniques have been described, each utilising different chemical properties of the components to be separated. Over the past 20 years, the introduction of HPLC using microparticulate stationary phases and large pressure gradients, has allowed the development of very rapid and sensitive methods for the separation of amino acids, peptides and proteins.

Most of the HPLC packing materials in current use are based on silica gels. Such gels consist of aggregates of spherical colloidal silica beads (2-20nm diameter) fused to give particles of 3-10 $\mu$ m diameter. This material is packed into stainless steel columns 10-25cm in length, with an internal diameter of 2-10mm.

Adsorption chromatography is one of the oldest liquid chromatographic techniques. Separation is based upon the selective

adsorption of the components of the mixture onto active sites on the surface of the stationary phase. Generally, a polar inorganic packing is used and in the case of silica, the most commonly used material, the active sites are hydroxyl groups. Organic solvents such as hexane or heptane are used to elute solutes from the column. Adsorption chromatography is not suitable for the chromatography of very hydrophobic molecules which are difficult to retain or of polar molecules, which are difficult to elute from the stationary phase.

To overcome these problems, packing materials with functional groups chemically bonded to the silica particles have been developed. The main types of functional group used in bonded phases are: i) hydrophobic groups especially octadecyl ( $C_{18}$ ) but also octyl ( $C_8$ ), ethyl ( $C_2$ ) and methyl ( $C_1$ ) ii) polar groups such as aminopropyl ( $NH_2$ ), cyanopropyl (CN) and glycol. These packing materials are prepared by reacting silanol groups on the silica particle surface with a silane derivatizing reagent containing the appropriate functional group.

In contrast to adsorption chromatography which uses a non-polar eluent, a polar eluent and hydrophobic or moderately polar packing materials such as  $C_{18}$ ,  $C_8$  or CN are used in reverse-phase chromatography. In this technique the mixture of solutes partitions itself between the stationary phase and the solvent. Maximal retention will occur when the solvent is water. As the concentration of organic solvent (most commonly methanol, acetonitrile or n-propanol) is increased, the solutes elute in decreasing order of polarity. Although hydrophobic interactions are the major factor in determining the order in which solutes



elute, other forces such as electrostatic attraction and hydrogen bonding play secondary roles. Reverse phase chromatography is suitable for the separation of polar molecules but non-polar compounds can also be successfully separated provided the proportion of organic solvent in the eluent is sufficient.

In addition to the column, an HPLC system consists of one or more pumps with fine flow rate adjustment and capable of operating pressures up to 6000 psi, an injection valve, a detection system, which may be electrochemical, fluorescence or absorbance and if necessary, a fraction collector.

HPLC offers a number of advantages over more conventional methods of protein and peptide separation. Separation time can be reduced from days to hours. HPLC systems can handle sample volumes from a few microlitres to hundreds of millilitres with detection in the picomole range and offer an increased selectivity compared to other methods. With a suitable choice of column, solvent composition and gradient such critical separations as the resolution of stereoisomers or of peptides differing by only a single amino acid can be achieved.

### 2.2.2 High Performance Liquid Chromatography of Somatostatin

#### i) Introduction

Since most radioimmunoassays for somatostatin are directed towards the C-terminal portion of the SS14 sequence, larger N-terminally extended forms of the peptide will also be detected. Total SSLI measured in any sample will, therefore, consist of contributions from the various N-terminally extended forms present

in the sample. Unless specific RIA's for SS28, SS6k and SS10k are available, chromatographic systems which distinguish between the various forms of SSLI are required in order that the tissue distribution of these larger peptides can be established.

The separation of multiple forms of SSLI by gel filtration chromatography has been extensively reported (Rorstad et al., 1979; Spiess & Vale, 1980; Patel et al., 1981; Benoit et al., 1982b). However, it is not possible to resolve SS28 and SS25 by gel-filtration chromatography. Additionally, in most reports, larger molecular forms of somatostatin elute at or near the void volume in poorly defined peaks.

A number of workers have confirmed the identity of peptides isolated by gel filtration chromatography by taking peak fractions and rechromatographing the material on HPLC (Corder et al., 1982; Gomez et al., 1983; Penman et al., 1983; Baldissera et al., 1985). These HPLC systems are not designed to separate one molecular form of somatostatin from another and do not detect HMW-SS.

Recently, an HPLC system has been described which appears to detect, in addition to SS14 and SS28, a higher molecular weight form of SS14-like immunoreactivity (Charpernet & Patel, 1985).

#### ii) Description of system

An HPLC system capable of resolving SS28 and SS14 was in use in the laboratory (Harman et al., 1982). This system used an Ultrasphere 5 $\mu$ m ODS column and a gradient of acetonitrile in sodium phosphate pH 2.1. However, high molecular weight forms of SSLI were not detected and it was not possible to separate SS28 and SS25. In order to establish if SS25 was present in tissues, or as

suggested by Esch et al. (1980) was an extraction artifact, a suitable HPLC separation had to be developed.

The HPLC system used in all studies consisted of an Altex 421 controller, with two Altex 110A pumps (Beckman Instruments, Berkeley, CA). This allows programmed gradients with variable flow rate, between two solvents. Samples were injected into the system via an Altex 210 injection valve which had in place of a sample loop, a disposable cartridge guard column of 5 $\mu$ m cyanopropyl silica (Spheri 5 Cyano: Brownlee Labs). This allowed samples to be loaded onto the system in acidified aqueous solvents. The main column was an Altex Ultrasphere 5 $\mu$ m ODS (25cm x 4.6mm internal diameter).

The detector used was a Hitachi model 100-10 spectrophotometer and was generally set at a wavelength of 225nm for detection of peptides with minimal interference from organic solvents. Samples were collected by an LKB 211 multirac fraction collector which can be programmed by the 421 controller to give up to three fraction sizes. For large sample volumes, a Milton Roy mini pump (Milton Roy Co., Riviera Beach FL.) was used to speed the loading of the sample onto the cartridge guard column.

A suitable resolution of SS28, SS25 and SS14 was obtained when a gradient from solvent A (0.2% (v:v) TFA in H<sub>2</sub>O pH 2.1) to solvent B (0.3% (v:v) TFA in acetonitrile) in three linear segments, 0.0% - 31% B over 5 min, 31% - 32.2% B over 12 min and 32.2% - 100% B over 15 min was used to elute the peptides from the column. The flow rate was 2ml min<sup>-1</sup>. In order to maximise resolution whilst keeping the number of fractions as small as possible, three different sizes were collected: 2ml fractions from 0.0 - 6.0ml and from 30.6 - 50.6ml, 1ml fractions from 6.0 - 9.0ml and from 20.6 -

30.6ml and 0.4ml fractions from 9.0 - 20.6ml. There was a nine minute delay after the sample was injected before the collection of fractions commenced. Samples were loaded directly onto the guard column and flushed through with solvent A before being placed in the solvent stream.

In addition to SS28 and SS14, high molecular weight forms of somatostatin were detected when tissue extracts were applied to the column. Synthetic SS28<sub>(1-12)</sub> eluted from the column prior to SS28. The elution positions of synthetic SS14, SS25, SS28 and SS28<sub>(1-12)</sub> are shown in figure 2.2.1. The elution position of HMW-SS is indicated. Fractions of three different sizes were collected for the periods shown.

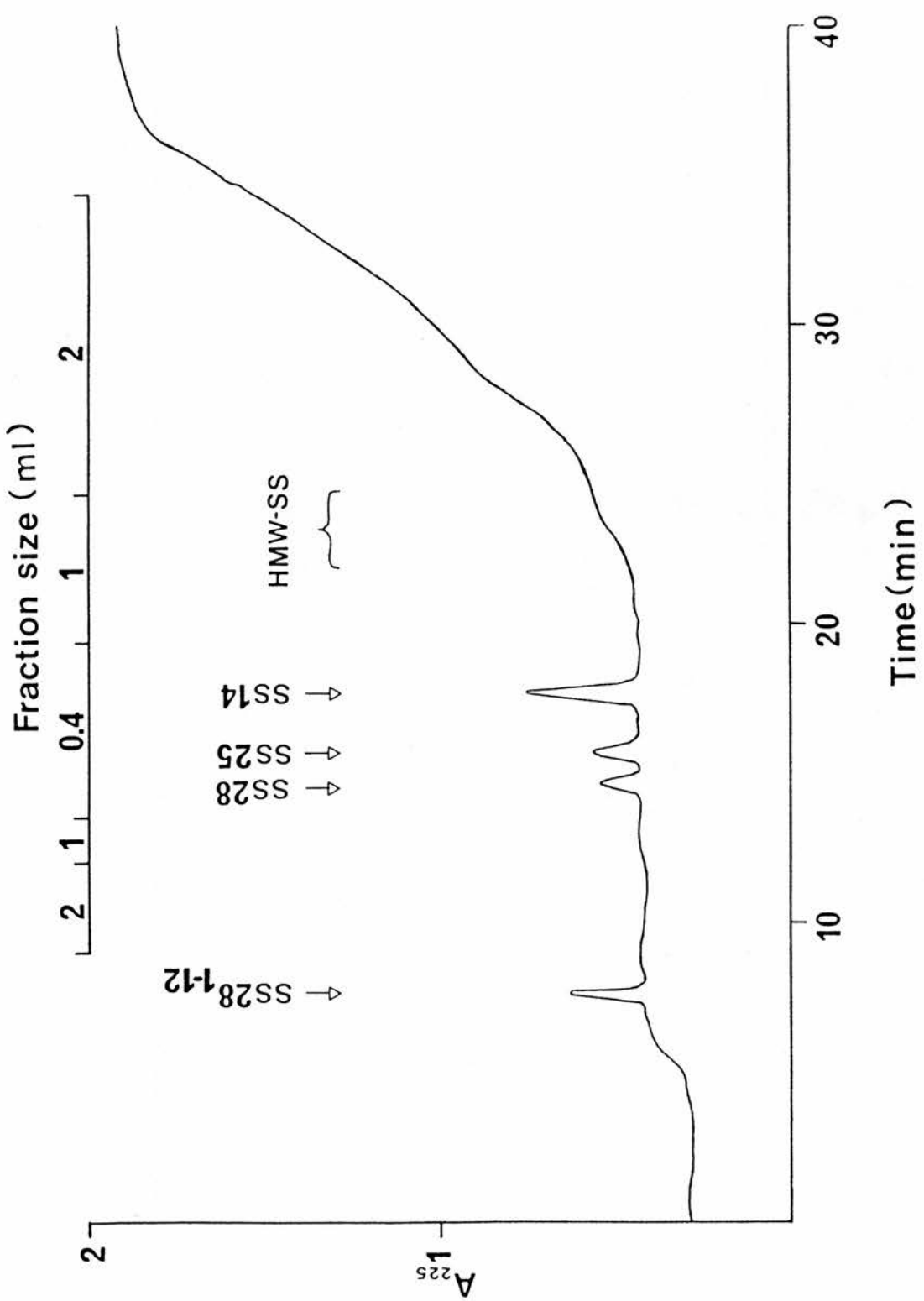
### iii) Comparison with other systems

The separation of multiple forms of SSLI using HPLC has many advantages over the use of gel filtration or ion-exchange chromatography. The HPLC system described here can resolve SS28, SS25 and SS14 as well as detecting larger forms of SSLI. Gel filtration cannot resolve SS28 and SS25 and requires large samples of tissue. In addition, gel filtration chromatography is considerably slower than HPLC.

The HPLC system described by Charpenet & Patel (1985) is similar to the system developed in this study. Both use columns packed with C<sub>18</sub> silica and the organic solvent used is acetonitrile. The system developed in this study has an important advantage over other chromatographic systems described for the separation of multiple forms of SSLI. A cartridge guard column is used in place of a conventional sample loop, allowing large volumes of dilute

Figure 2.2.1 Elution of synthetic SS14, SS25, SS28 and SS28(1-12) from HPLC

A representative chromatogram of the elution positions of synthetic SS28, SS25, SS14 and SS28(1-12) is shown. HMW-SS obtained from tissue extracts eluted in the position shown. Fractions of three different sizes (2ml, 1ml, 0.4ml) were collected for the periods indicated. 5ug of each peptide were applied to the column.



somatostatin-containing perifusate or plasma to be desalted and concentrated without the need for either extraction or freeze-drying, thus minimising losses which may occur by either of these methods. Additionally, tissue homogenates prepared in acetic acid can be loaded directly onto the cartridge without prior freeze drying.

CHAPTER 3

DISTRIBUTION OF MULTIPLE FORMS OF SOMATOSTATIN-LIKE  
IMMUNOREACTIVITY IN THE RAT BRAIN



### 3.1 INTRODUCTION

In different tissues, a single polypeptide precursor molecule may be processed to generate different patterns of biologically active products. The best known example is proopiomelanocortin (POMC), the common precursor to ACTH, the melanocyte stimulating hormones ( $\alpha$ -  $\beta$ - and  $\gamma$ -MSH) and the endorphin family of opioid peptides (Nakanishi et al., 1979). In the anterior pituitary, the predominant products of POMC processing are ACTH and  $\beta$ -endorphin, whereas in the pars intermedia  $\alpha$ MSH, corticotrophin-like intermediate lobe peptide (CLIP) and acetylated, biologically inactive forms of endorphin are produced (Smyth & Zakarian, 1980). Evidence is now accumulating to suggest that the somatostatin precursor may also be susceptible to tissue-specific post-translational processing.

It is well established that SSLI in rat brain is heterogeneous, consisting of in addition to SS14 and SS28, 6,000 and 10,000 mol. wt. species (Benoit et al., 1982b). A number of reports indicate that the relative abundance of these multiple forms of SSLI may vary in different tissues. SS14 is the most abundant form of the peptide in most regions of the CNS (Patel et al., 1981; Gomez et al., 1983). In the gastrointestinal tract, SS14 is the most abundant form of SSLI in the pylorus, duodenum and pancreatic D cell, but in the mucosal cells of the ileum and colon, SS28 predominates (Penman et al., 1983; Baskin & Ensink, 1984).

Although in anglerfish there are two somatostatin precursors (Hobart et al., 1980), in mammals, only one has been found (Shen et al., 1982; Goodman et al., 1983). The different patterns of multiple forms of SSLI seen in the various tissues of the rat, must

arise from differential post-translational processing of this single precursor.

An immunocytochemical demonstration of differential processing of proSS in human intestine and pancreas has been achieved using specific antisera to SS28 and SS28<sub>(1-12)</sub>. Ravazzola et al. (1983), have shown that in the pancreatic D cell (which contains SS14), maturing secretory granules contain SS28 immunoreactivity but mature granules contain only SS28<sub>(1-12)</sub> immunoreactivity. In intestinal mucosa (an SS28 containing tissue) both maturing and mature granules exhibit an equally high staining with a SS28 specific antiserum. In the SS14-producing tissue SS28 is cleaved in the developing granule into SS14 and SS28<sub>(1-12)</sub>. Such a cleavage does not take place in an SS28-containing tissue.

SS28<sub>(1-12)</sub> has been shown by immunocytochemistry to be located in the same cells as SS28 and SS14 in the hypothalamus (Guy et al., 1985), suggesting that SS28 is the biosynthetic precursor to both SS14 and SS28<sub>(1-12)</sub> in these cells. Additionally, studies of somatostatin biosynthesis in the dorsal root ganglia (Harmar et al., 1982) have shown that SS28 is an intermediate in the biosynthesis of SS14. However, the presence of larger forms of SS28<sub>(1-12)</sub>-like immunoreactivity in rat hypothalamus and extra hypothalamic brain suggests that SS14 may be produced directly from the precursor and not via SS28 (Benoit et al., 1982b; Wu et al., 1983; Patel, 1983). These two seemingly contradictory results could be explained if the somatostatin precursor was subject to tissue specific post-translational processing.

A number of reports suggest that a different pattern of multiple forms of SSLI may be present in neurones projecting to the median

eminence than in the rest of the hypothalamus. SS28 has been detected in equimolar quantities with SS14 in extracts of hypophysial portal blood (Millar et al., 1983) and is released by rat median eminence synaptosomes (Kewley et al., 1981). Additionally, SS14 and SS28 are reported to be present in the neurohypophysis of the rat in approximately equimolar amounts (Gomez et al., 1983). In order to establish whether the processing of proSS in neurones projecting to the median eminence is different from that in the rest of the hypothalamus, the regional distribution of molecular forms of SSLI in the amygdala and hypothalamus of the rat has been examined by HPLC and RIA.

### 3.2 METHODS

#### 3.2.1 Extraction of SSLI from tissues

Male COB Wistar rats (180-230g body weight), purchased from Charles River U.K. Ltd. (Margate, Kent), were maintained under controlled lighting (09.00-19.00h) and temperature (22°C) and allowed free access to Diet 41B (Oxoid, Basingstoke, Hants) and tap water. Within 1-2 weeks of arrival in the laboratory, the animals were killed by decapitation, the brains rapidly removed and transferred to ice-cold NaCl (0.9%, w:v). After 1-2 min the brains were placed on filter paper with the ventral surface uppermost. The pituitary stalk was grasped with watchmaker's forceps and the median eminence, defined as the vascularized area approximately 0.5mm wide and 2-3mm long lying at the floor of the third ventricle, was dissected out with iridectomy scissors. Two coronal cuts were then made, one immediately rostral to the optic chiasm and the other

caudal to the mammillary bodies. Hypothalamus and amygdala were dissected from the block of tissue isolated by the two cuts. The hypothalamic sample extended from the level of the anterior commissure ventrally, to the hypothalamic sulci laterally. Material lying ventrally to the anterior commissure and laterally to the hypothalamus was taken as the amygdala sample. Tissue wet weights were determined before the fragments were homogenized in 10 volumes of ice-cold 2M acetic acid using an all-glass homogenizer.

After removal of 50 $\mu$ l of the homogenate for protein determination, extracts were centrifuged (8000g for 10 min), the supernatant was removed, a 50 $\mu$ l aliquot taken for RIA of total SSLI, and the remainder was passed through a Millex GV disposable filtration unit (Millipore Corp., Bedford, MA) before chromatography. The recovery of SSLI through the filter unit was  $85 \pm 6\%$  (mean  $\pm$  S.D. n = 10).

### 3.2.2 Protein determination

Protein was determined by the Coomassie Blue G-250 binding method of Bradford (1976) supplied in kit form (Bio-Rad Laboratories, Richmond, CA), using bovine serum albumin as standard. 1-10 $\mu$ l aliquots of homogenate were assayed in duplicate.

### 3.2.3 Gel filtration chromatography

Gel filtration chromatography was performed on a column of Sephadex G-75 (Superfine, 85cm x 1.6cm) equilibrated with 30% acetic acid, and calibrated using cytochrome c (mol. wt. 12,300),  $^{125}$ I-labelled insulin (mol. wt. 5,700) and substance P (mol. wt. 1,348). The exclusion volume determined using dextran blue 2000

was 34.6ml. The solvent used for elution was 30% acetic acid and flow rate was  $5\text{ml h}^{-1}$ . The extract from nine hypothalami was freeze-dried, resuspended in 1.5ml 30% acetic acid and applied to the column. Fractions (1ml) were collected and 100 $\mu$ l aliquots dried in a vacuum oven before assay for SSLI as described in section 2.1. 10-50 $\mu$ l aliquots of peak fractions were reassayed in duplicate.

#### 3.2.4 HPLC and RIA

The filtered sample was loaded onto the guard column of the HPLC and eluted as described in section 2.2. After HPLC, the 400 $\mu$ l fractions were made up to 1ml by the addition of  $\text{H}_2\text{O}/0.2\%$  TFA and 200 $\mu$ l aliquots of eluate were dried in a vacuum oven prior to RIA. Peak fractions were reassayed in duplicate at three dilutions within the range of the assay (20-100 $\mu$ l) in order to quantitate SSLI present. 0.1-10 $\mu$ l aliquots of unfiltered supernatant were assayed in duplicate to determine total SSLI.

### 3.3 RESULTS

Gel filtration chromatography of hypothalamic extracts revealed the presence of four forms of SSLI with estimated molecular weights of 1,500, 3,000, 6,000 and 10,000 (figure 3.3.1). Rechromatography of each of these peaks by HPLC showed that the 1,500 and 3,000 mol. wt. material co-eluted with synthetic SS14 and SS28 respectively. The 6,000 and 10,000 mol. wt. forms of SSLI were not completely resolved from one another on HPLC and eluted at a position later than SS14, SS25 and SS28 between 25.6 and 30.6ml as a composite peak



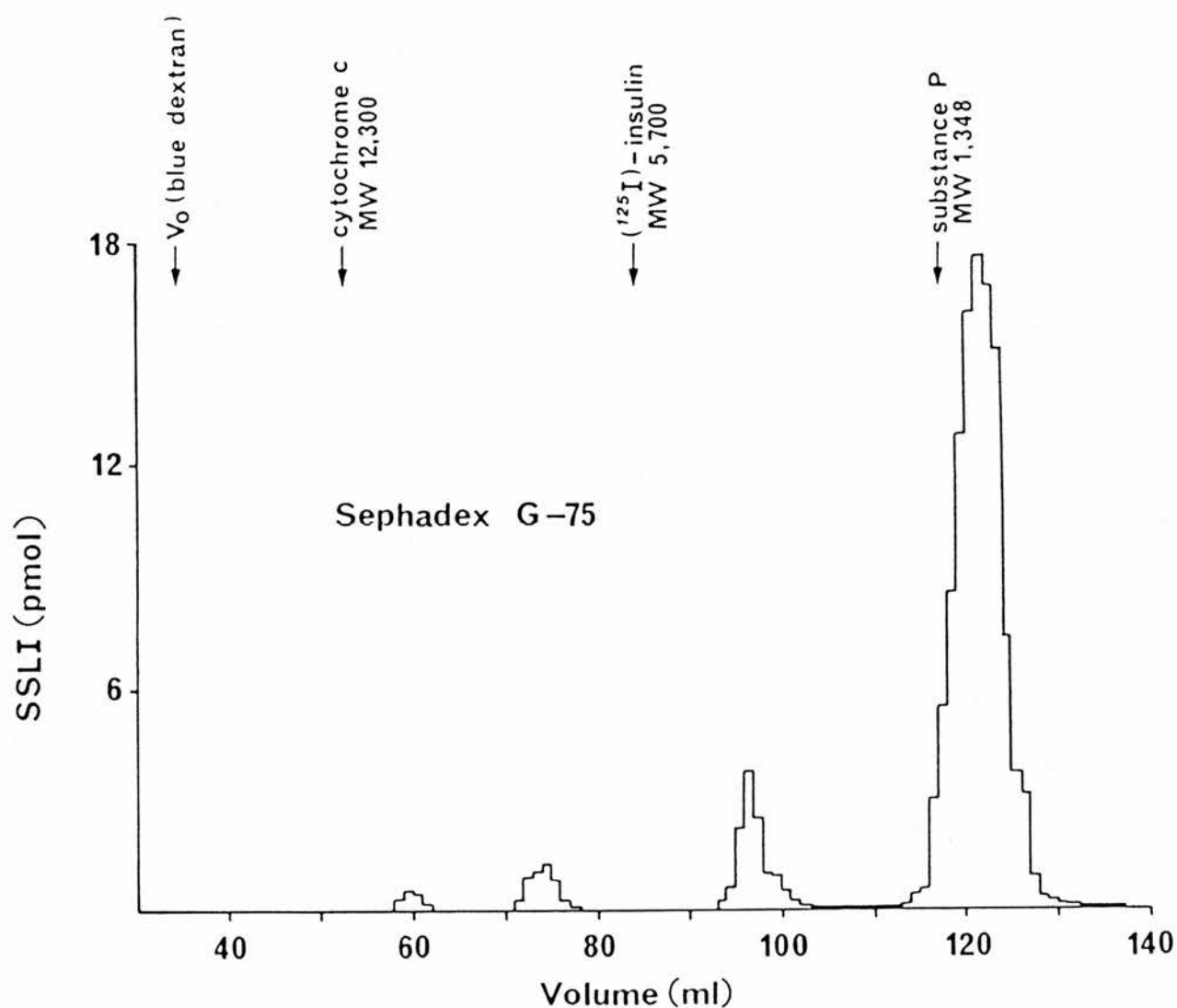


Figure 3.3.1 Elution profile of SSLI extracted from the rat hypothalamus

An extract of nine rat hypothalami was subjected to Sephadex G-75 chromatography as described in section 3.2.3. Arrows indicate the elution positions of markers used to calibrate the column.

of high molecular weight somatostatin (HMW-SS).

The elution profiles of SSLI after HPLC of extracts of hypothalamus, median eminence and amygdala are shown in figure 3.3.2. The three tissues contained peaks of SSLI corresponding to SS14, SS28 and HMW-SS. There was no evidence for the presence of a peptide with the chromatographic properties of SS25. Chromatographic profiles obtained using amygdala and hypothalamus were comparable, whereas the median eminence contained a much larger proportion of SS28 than the other tissues. The mean content of each peak of SSLI in the three regions (based on six separate HPLC separations performed on samples of each region) is shown in Table 3.1. In amygdala and hypothalamus, the predominant form of SSLI present was SS14, accounting for 60% of total SSLI, 9-12% of the SSLI corresponded to SS28, with the remainder present as HMW-SS. In contrast, the median eminence contained approximately equimolar amounts of SS14 and SS28 (40% and 34% respectively) and smaller amounts of HMW-SS.

### 3.4 DISCUSSION

Gel filtration chromatography showed that rat hypothalamic extracts contain four forms of SSLI (SS14, SS28 and 6,000 and 10,000 mol. wt. species). High performance liquid chromatography of extracts of the amygdala indicated that the profile of immunoreactive forms of somatostatin present was similar to that observed in the hypothalamus, with the predominant form being SS14. In contrast, the median eminence contained a much larger proportion of SS28 (34%) than the other tissues. SS25 was not

Figure 3.3.2     HPLC analysis of SSLI extracted from the  
rat Hypothalamus, amygdala and median  
eminence

Chromatography and RIA were performed as described in Chapter 2. The elution positions of synthetic SS28, SS25, SS14 and of HMW-SS are indicated. Results are expressed as picomoles SSLI per millileter of HPLC eluate; representative profiles for each of the tissues are shown.



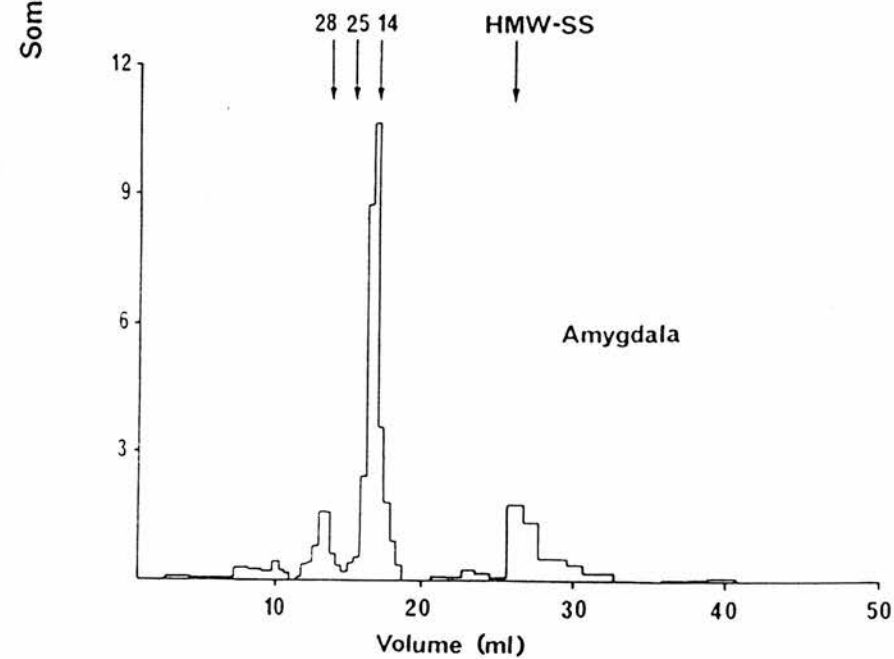
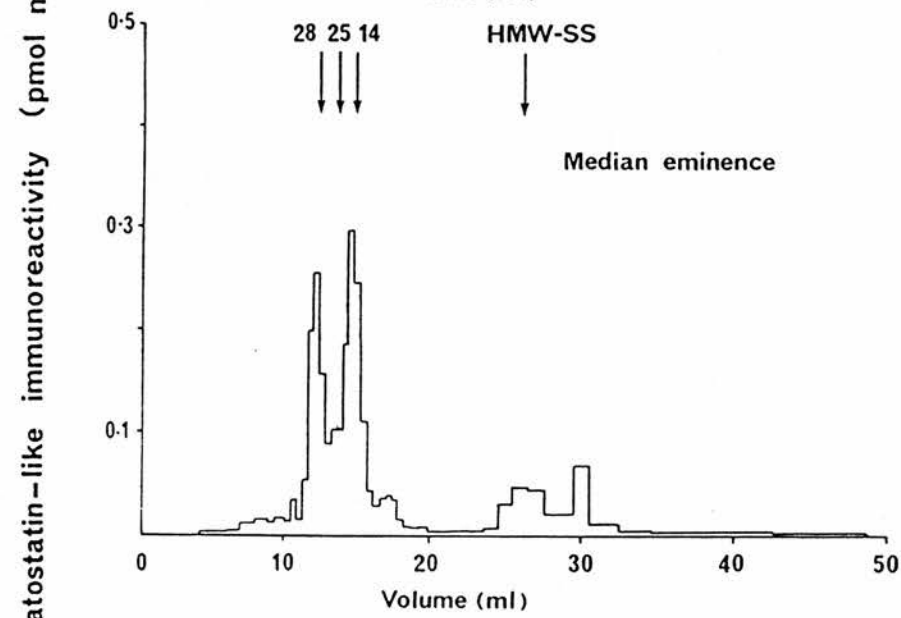
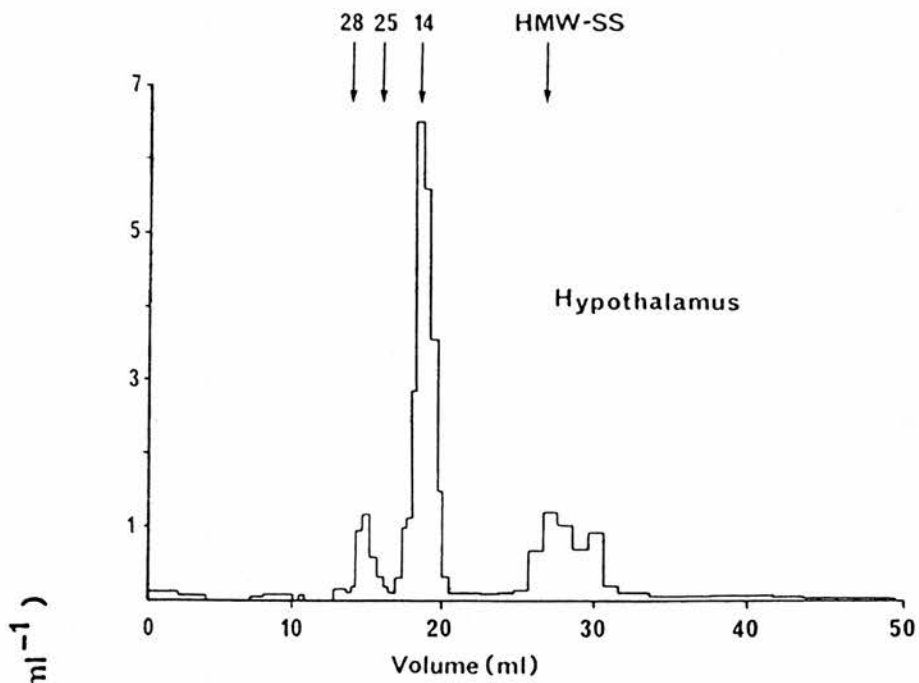


TABLE 3.1

## DISTRIBUTION AND HETEROGENEITY OF SSLI IN RAT BRAIN REGIONS

Regions (No. of determinations)	Total SSLI pmol mg protein <sup>-1</sup>	Proportions of molecular forms (%)		
		SS14	SS28	HMW-SS
Amygdala (6)	23.0 $\pm$ 1.6	63 $\pm$ 7	12 $\pm$ 2	25 $\pm$ 10
Hypothalamus (6)	17.4 $\pm$ 1.4	59 $\pm$ 8	9 $\pm$ 1	32 $\pm$ 4
Median Eminence (12)	205.6 $\pm$ 24.2	40 $\pm$ 2	34 $\pm$ 2	26 $\pm$ 4

Tissue extracts were prepared as described in section 3.2.1. Estimates of total SSLI were based upon duplicate determinations from four dilutions of each sample; all samples were within the range of the assay and displacement curves were parallel with that of synthetic SS14. Tissue extracts were subjected to HPLC analysis as described in section 3.2.4. Total amounts of SS14, SS28 and HMW-SS are expressed as a percentage of total SSLI. Values are mean  $\pm$  s.e.m. The number of determinations is shown in parenthesis.

detected in tissue extracts and as suggested by Esch et al. (1980), it was possible that the SS25 isolated from ovine hypothalamus was generated artefactually from SS28 during purification.

The presence of four forms of SSLI with molecular weights similar to those reported here have been described in rat brain (Benoit et al., 1982b) and in human phaeochromocytoma tissue (Wu et al., 1983). The 10,000 mol. wt. form of SSLI probably corresponds to the rat prosomatostatin sequence (mol. wt. 10,400) deduced from the nucleotide sequence of cloned cDNA by Goodman et al. (1983). SS6k may be an intermediate in the post-translational processing of proSS into SS28 and SS14.

In agreement with Gomez et al. (1983), SS14 was the predominant form of SSLI observed in the hypothalamus and amygdala. However, when the median eminence was examined, a different profile of molecular forms of SSLI was observed. The median eminence contained approximately equimolar amounts of SS28 and SS14.

The distribution of multiple forms of SSLI has been studied in a number of tissues. In most parts of the CNS, SS14 is the predominant form of SSLI present, accounting for at least 60% of the total immunoreactivity (Patel et al., 1981; Gomez et al., 1983). The rat retina and sciatic nerve contain predominantly SS14 with little or no larger material present (Patel et al., 1981). In the gastrointestinal tract, human pylorus and pancreas contain greater than 90% SS14 (Penman et al., 1983). However, other parts of the intestinal tract contain a greater proportion of SS28 than SS14. In the mucosal layer of the colon, ileum and in the body of the stomach in man, SS28 predominates (Penman et al., 1983). Baskin & Ensink (1984) have shown by immunocytochemistry that SSLI in the

epithelial cells of the intestinal mucosa is present primarily as SS28 whereas in other cells such as the pancreatic D cell only SS14 is present. A third type of tissue has been observed which contains both SS14 and SS28 in comparable amounts. The rat neurohypophysis (Gomez et al., 1983), rat epididymis (Peckary et al., 1984) and human lumbar CSF (Beal et al., 1985b), contain similar proportions of SS14 and SS28. The regional differences in peptide distribution are presumably due to differential processing of the somatostatin precursor in these tissues.

These findings suggest that there are at least two types of somatostatin producing cell type: SS14-producing and SS28-producing. Tissues which contain predominantly one form of SSLI will be innervated by the appropriate type of neurone. Those tissues which contain both SS14 and SS28 may be innervated by both SS14- and SS28-producing neurones or may contain a third type of neurone which produces both forms of the peptide.

The specificity of somatostatin-producing neurones could be achieved by the differential distribution of the somatostatin processing enzymes within each type of neurone. The generation of SS14 from the precursor will depend on the activity of at least two enzymes, i) the "SS28 convertase" (Gluschankof et al., 1984), ii) an enzyme with a specificity for a single arginyl residue generating SS28 and possibly iii) an enzyme with a specificity for pairs of basic amino acids which is not the "SS28 convertase", which cleaves SS14 directly from the precursor or from SS6k.

In a SS14-producing neurone, proSS is cleaved first into SS28 by the enzyme with a specificity for single arginyl residues and then subsequently into SS14 by the "SS28 convertase". If the activity

of the SS28 generating enzyme was rate limiting, all the SS28 produced would be converted into SS14. Alternatively, SS14 could be cleaved directly from the precursor without SS28 as an intermediate. In an SS28-producing neurone, proSS will be cleaved into SS28 by the enzyme with a specificity for single arginyl residues. The "SS28 convertase" may be absent or inactive. In this way SS28 could not be cleaved into SS14. In neurones which produces both SS14 and SS28, the activity of the "SS28 convertase" may be such that only a limited conversion of SS28 into SS14 takes place. For a complete understanding of the tissue specific processing of proSS in neurones and endocrine cells, further studies of the distribution and properties of the processing enzymes will be required.

The results in this chapter provide evidence that the neurones projecting to the median eminence differ from those in the rest of the hypothalamus in their pattern of proSS processing. It is probable that SS28 plays a neurohormonal role confined to the median eminence but that SS14 is the predominant form of SSLI involved in neurotransmission in the rest of the hypothalamus. The physiological significance of this result will be discussed in the next chapter.

## CHAPTER 4

RELEASE OF MULTIPLE FORMS OF SOMATOSTATIN-LIKE IMMUNOREACTIVITY  
FROM THE RAT HYPOTHALAMUS IN VITRO

#### 4.1 INTRODUCTION

The secretion of pituitary hormones is under the control of a number of releasing and release inhibiting factors released into hypophyseal portal vessel blood by the median eminence. A number of these factors have been isolated and characterised and have been shown to be peptides ranging from 3 to 44 amino acids in length. The releasing hormones for GH, TSH, ACTH and the gonadotrophins have all been identified and the cDNA sequences of their precursors have been established (Gubler et al., 1983; Mayo et al., 1983; Lechan et al., 1986; Furutani et al., 1983; Seeburg & Adelman, 1984).

The first peptide inhibitory hormone, somatostatin, was identified in hypothalamic extracts (Brazeau et al., 1973): the cDNA sequence of the precursor has been established (Shen et al., 1982; Goodman et al., 1983). Recent work (Nikolics et al., 1985) suggests the presence of a second peptide inhibitory factor (prolactin release-inhibiting factor) within the sequence of the gonadotrophin releasing hormone precursor.

In addition to inhibiting GH and TSH secretion from the pituitary gland, somatostatin inhibits both insulin and glucagon secretion from the pancreas and the release of a number of hormones in the gastrointestinal tract. No function has yet been ascribed to somatostatin in CNS neurones but it has been postulated to act as a neurotransmitter. An important criterion for identification of a neurotransmitter is the demonstration of release of the substance from nerve terminals in a  $\text{Ca}^{2+}$  dependent manner in response to membrane depolarization. SSLI can be released in response to a depolarizing stimulus of high  $\text{K}^{+}$  concentration from rat hypothalamic slices (Iversen et al., 1978), posterior pituitary

(Patel et al., 1977), spinal cord (Sheppard et al., 1979) and from cultured foetal hypothalamic cells in long term culture (Gamse et al., 1980; Peterfreund & Vale, 1982). Little information is available concerning the molecular forms of SSLI released from the hypothalamus, although there is evidence for the release of larger forms of SSLI from cultured cells (Peterfreund & Vale, 1982). The release of SS28<sub>(1-12)</sub> LI has been observed in rat hypothalamic slices (Bakhit et al., 1983).

Chromatographic analysis of extracts of hypophysial portal vessel blood (Millar et al., 1983) have shown that SS28 is present in quantities equal to or greater than SS14 and a depolarizing stimulus releases both SS14 and SS28 from synaptosomes prepared from ovine median eminence (Kewley et al., 1981). The results of Chapter 3 have shown that in the hypothalamus SSLI is heterogeneous, with a greater proportion of SS28 being found in the median eminence compared with the rest of the hypothalamus. These findings taken together with a report that SS28 is more potent than SS14 in inhibiting GH secretion in vitro (Brazeau et al., 1981), suggest that SS28 may play a role in the inhibition of pituitary growth hormone release in vivo.

In order to provide further evidence for a physiological role for SS28 in the median eminence, the molecular forms of SSLI released basally and in response to a depolarizing stimulus of K<sup>+</sup> from the isolated median eminence have been examined and compared to those released from hypothalamic slices.



## 4.2 METHODS

### 4.2.1 Preparation of tissues

Male COB Wistar rats (180–230g body weight) purchased from Charles River U.K. Ltd. (Margate, Kent), were maintained under controlled lighting (0900–1900h) and temperature (22°C) and allowed free access to Diet 41B (Oxoid, Basingstoke, Hants) and tap water. Within 1–2 weeks of arrival in the laboratory, the animals were killed by decapitation and the brain removed. Hypothalamic tissue was dissected as described in section 3.2.1 and sliced in the sagittal plane at 250µm intervals using a McIlwain tissue chopper. Median eminence tissue was removed from the intact brain as described in section 3.2.1. Hypothalamic slices and median eminences were placed in a modified Krebs–bicarbonate medium of the following composition.

	mM
Na Cl	127
K Cl	3.83
Ca Cl <sub>2</sub>	1.8
KH <sub>2</sub> PO <sub>4</sub>	1.18
Mg SO <sub>4</sub>	1.18
Na HCO <sub>3</sub>	20
D-glucose	11.1
Bovine serum albumin	1mg ml <sup>-1</sup>
Bacitracin	30µg ml <sup>-1</sup>
Trasylo1	400 KIU ml <sup>-1</sup>

#### 4.2.2 Perifusion of tissues

The perifusion apparatus consisted of four perspex perifusion chambers with a nylon gauze onto which hypothalamic slices or median eminence tissue were placed (Jessell et al., 1976). The chambers were placed in a water bath at 37°C. The medium was gassed with an oxygen:carbon dioxide (95:5% v:v) mixture and pumped at a flow rate of  $0.3\text{ml min}^{-1}$  with a multi-channel peristaltic pump. After an initial wash period of 30 minutes to allow tissues to equilibrate, samples were collected for 3 minutes periods into polypropylene tubes containing 0.5ml aqueous TFA (3% v:v). In order to depolarize tissues, a three minute pulse of medium containing an additional 50mM KCl was given. In some experiments, a calcium free medium in which  $\text{Mg}^{2+}$  was substituted for  $\text{Ca}^{2+}$  was used.

#### 4.2.3 Estimation of SSLI

Two types of experiment were performed, i) determination of total SSLI released ii) identification of multiple forms of SSLI released. In experiments measuring total SSLI released, slices from a single hypothalamus or six intact median eminences were placed in each of the four perifusion chambers. Extraction of total SSLI in each 3 minute fraction was carried out using disposable columns of  $\text{C}_{18}$  (Baker-10 ODS 1ml: Baker Chemical Co., Philipsburg, NJ). Columns were preconditioned by washing with 2ml of TFA-methanol (1% v:v) followed by 2ml of aqueous TFA (1% v:v). Samples were loaded onto the column and washed with 2ml aqueous TFA to remove salts. The peptides were eluted from the column with 1ml of TFA-methanol. The recovery of synthetic SS14 and SS28 (20ng) applied to these columns was  $88 \pm 3\%$  (mean  $\pm$  S.D.). Samples were

evaporated to dryness in a vacuum oven before determination of SSLI by radioimmunoassay as described in section 2.1. After each experiment the tissues were removed from the perfusion chambers and homogenised in 2M acetic acid before determination of total tissue SSLI. In these experiments, SSLI release was expressed as a rate constant: percentage of the total SSLI content of the tissue released per minute.

Slices obtained from eight hypothalami or sixty median eminences were used to determine the molecular forms of SSLI released. The perfusates from three nine minute periods: i) before the depolarizing stimulus ii) during and immediately after  $K^+$  stimulation and iii) once release levels had returned to normal, were combined and subjected to HPLC. Each sample was loaded directly onto the cartridge guard column in the sample loop of the HPLC using a pump. In this way, the sample was concentrated and desalted without the need for freeze drying. HPLC and RIA were carried out as described in Chapter 2.

#### 4.3 RESULTS

##### 4.3.1 Calcium-dependent release of SSLI

In normal Krebs medium SSLI was released from hypothalamic slices with a rate constant of less than  $0.02\% \text{ min}^{-1}$  (figure 4.3.1). Addition of 50mM KCl to the medium caused an immediate increase of 15-20 fold in the rate of release of SSLI. Basal release of SSLI was diminished and  $K^+$ -evoked release virtually abolished when  $Mg^{2+}$  was substituted for  $Ca^{2+}$  in the perfusion medium (figure 4.3.1). The basal release rate of SSLI from the

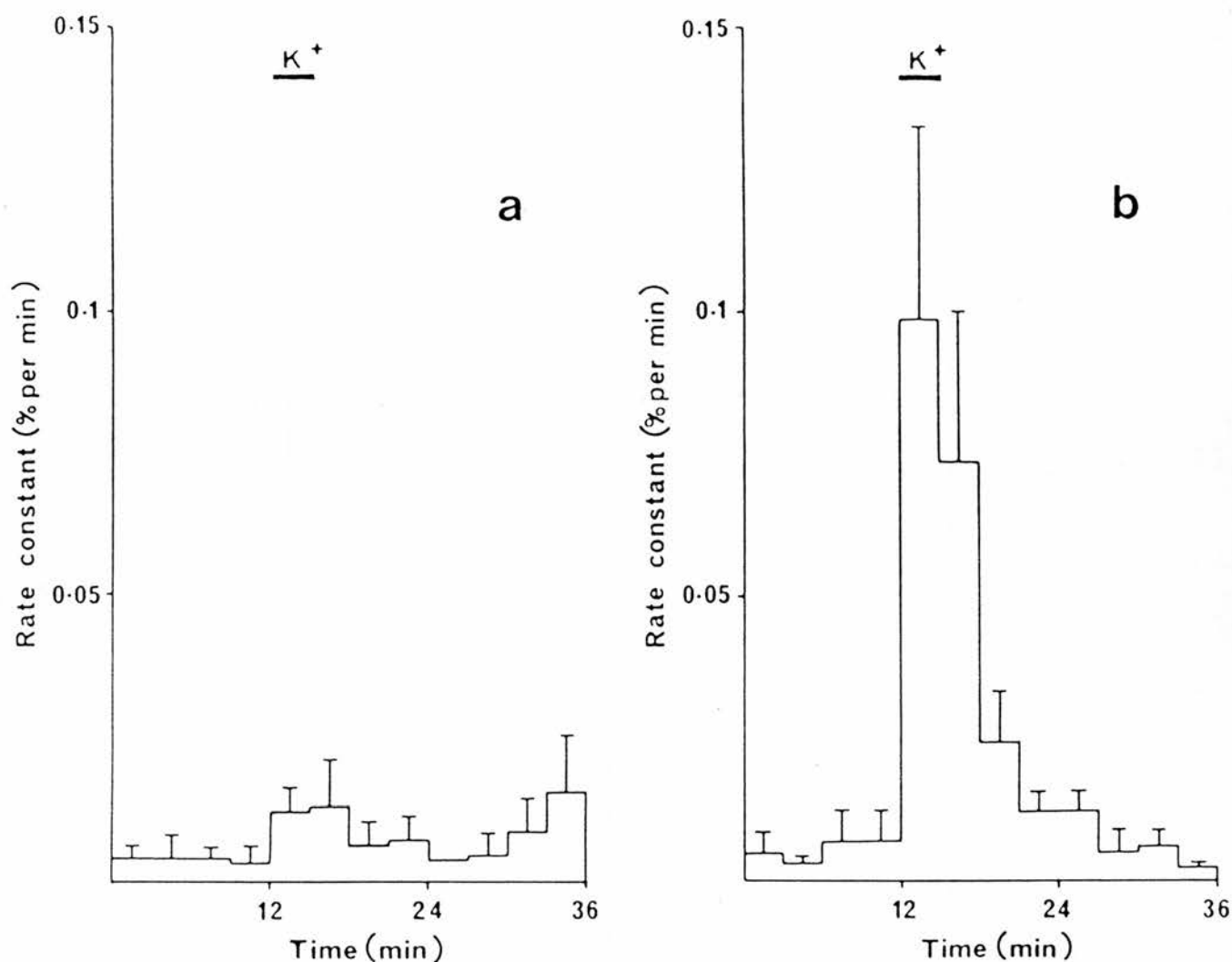


Figure 4.3.1 Release of SSLI from hypothalamic slices in vitro

Hypothalamic slices were perfused with a modified Krebs-bicarbonate medium and SSLI released was measured by RIA as described in section 2.1. Tissues were exposed to a depolarizing pulse of KCl (50mM) for the periods indicated a)  $Mg^{2+}$  substituted for  $Ca^{2+}$  in the medium throughout the experiment b) hypothalamic slices, normal Krebs medium. Results are expressed as percentage total tissue SSLI released per minute based on measurement of the tissue content at the end of the perfusion (mean  $\pm$  S.E.M. for four experiments).

Total SSLI release from hypothalamic slices was performed by Ms. L.A. Tannahill

isolated median eminence was  $0.002-0.007\% \text{ min}^{-1}$  and depolarization increased the rate of SSLI by approximately 7 fold (figure 4.3.2).  $\text{K}^+$  evoked release was abolished in calcium-free medium.

Comparison of tissue SSLI content at the end of the experiment with freshly dissected tissue showed that the SSLI released represented only a small proportion of the total SSLI in the tissue. This allowed release rate constants to be expressed as a percentage of total SSLI content of the tissue after the experiment.

#### 4.3.2 Determination of multiple forms of SSLI by HPLC

Perifusates from hypothalamic slices contained two major peaks of SSLI eluting in the same positions as SS14 and SS28 (figure 4.3.3). During a pulse of 50mM KCl 70% of the SSLI released corresponded to SS14 with a further 17% present as SS28.  $\text{K}^+$  depolarization increased the rate of release of SS14 by 12-fold but did not significantly increase the rate of release of SS28. In contrast perifusates from isolated median eminence tissue depolarised with 50mM  $\text{K}^+$  contained SS14, SS28 and HMW-SS (figure 4.3.4). Fifty-eight per cent of the SSLI released during the depolarizing stimulus was SS14, with a further 25% corresponding to SS28. The remainder (17%) corresponded to HMW-SS. The rates of SS14, SS28 and HMW-SS release were increased by 4.3, 5.5 and 1.6 fold respectively.

In both hypothalamic and median eminence tissue, the nine minute sample after the  $\text{K}^+$  stimulus showed that the proportion of each form of SSLI returned to basal levels values after  $\text{K}^+$  depolarization.

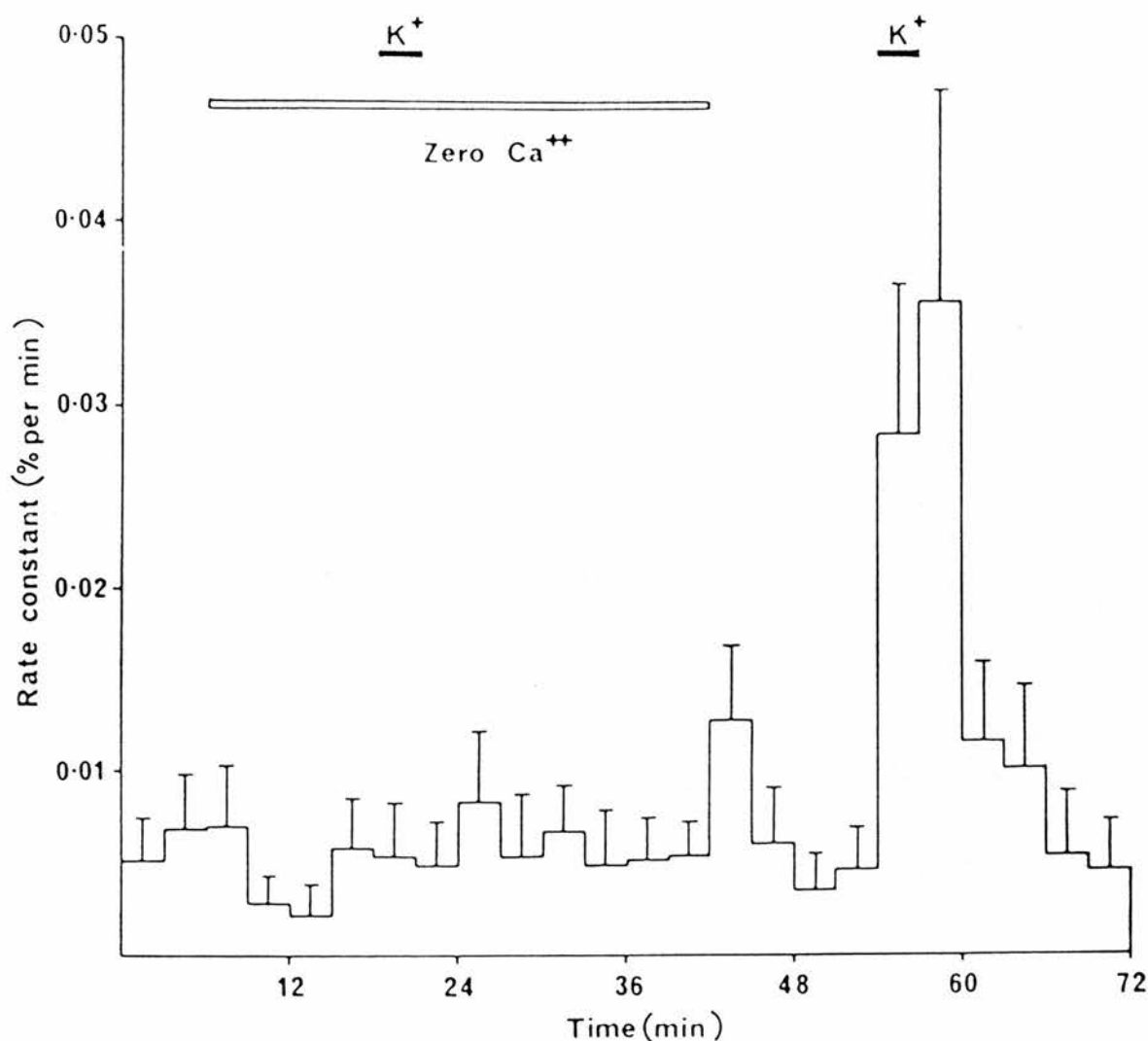


Figure 4.3.2 Release of SSLI from the isolated median eminence in vitro

Median eminences were perfused with a modified Krebs-bicarbonate medium and SSLI released was measured by RIA as described in section 2.1. Tissues were exposed to a depolarizing pulse of KCl (50mM) for the periods indicated.  $Mg^{2+}$  was substituted for  $Ca^{2+}$  during the period of zero  $Ca^{2+}$ . Results are expressed as percentage total tissue SSLI released per minute based on measurement of the tissue content at the end of the perfusion (mean  $\pm$  S.E.M. for four experiments).

Figure 4.3.3     HPLC analysis of SSLI released from  
hypothalamic slices in vitro

Perifusate samples from 8 hypothalami were collected for nine minute periods a) before b) during and c) after exposure of tissue to a depolarizing pulse of KCl. HPLC analysis and RIA were performed as described in Chapter 2. Results are expressed as femtomoles SSLI per milliliter of HPLC eluate; the elution positions of synthetic SS28, SS14 and of HMW-SS are indicated.

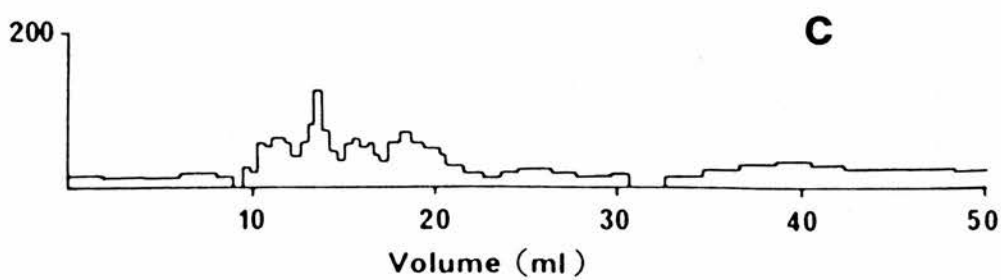
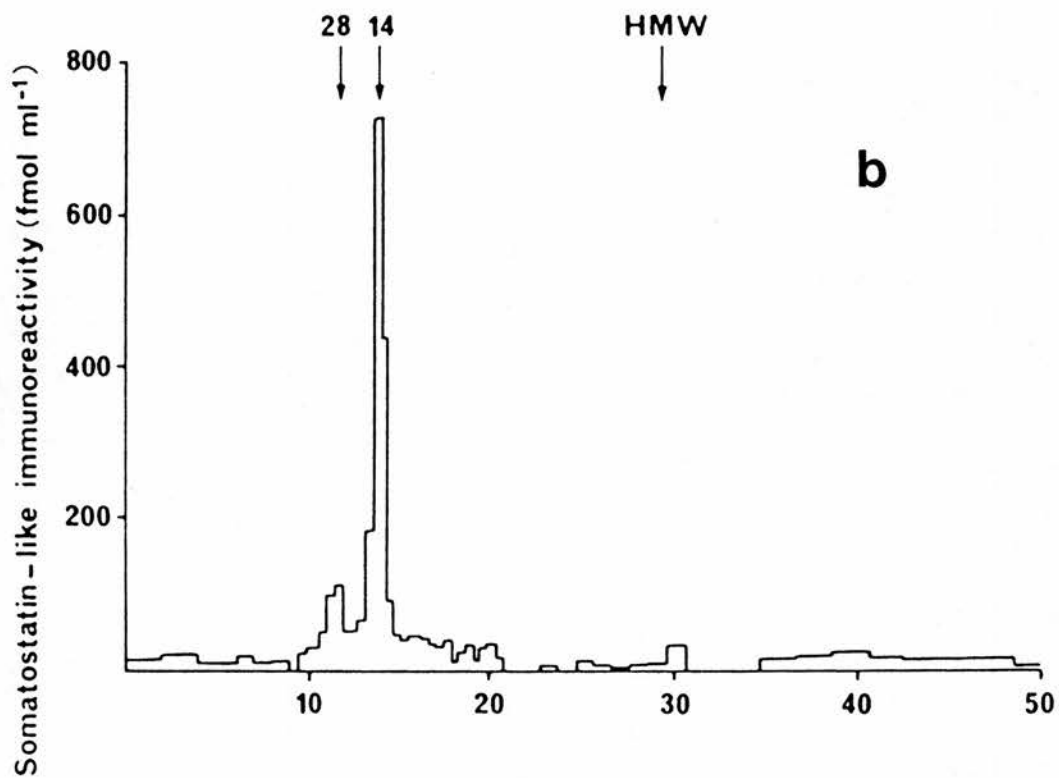
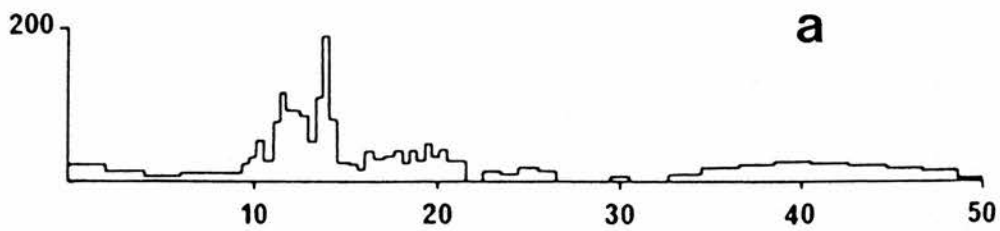
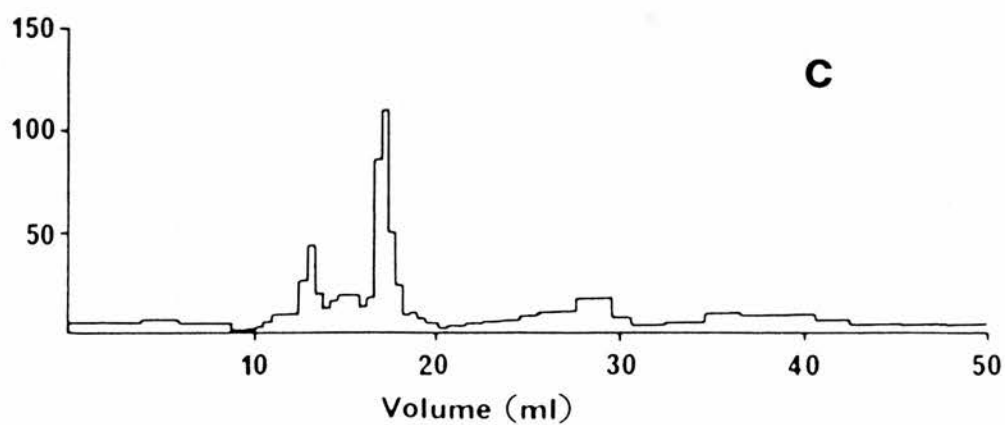
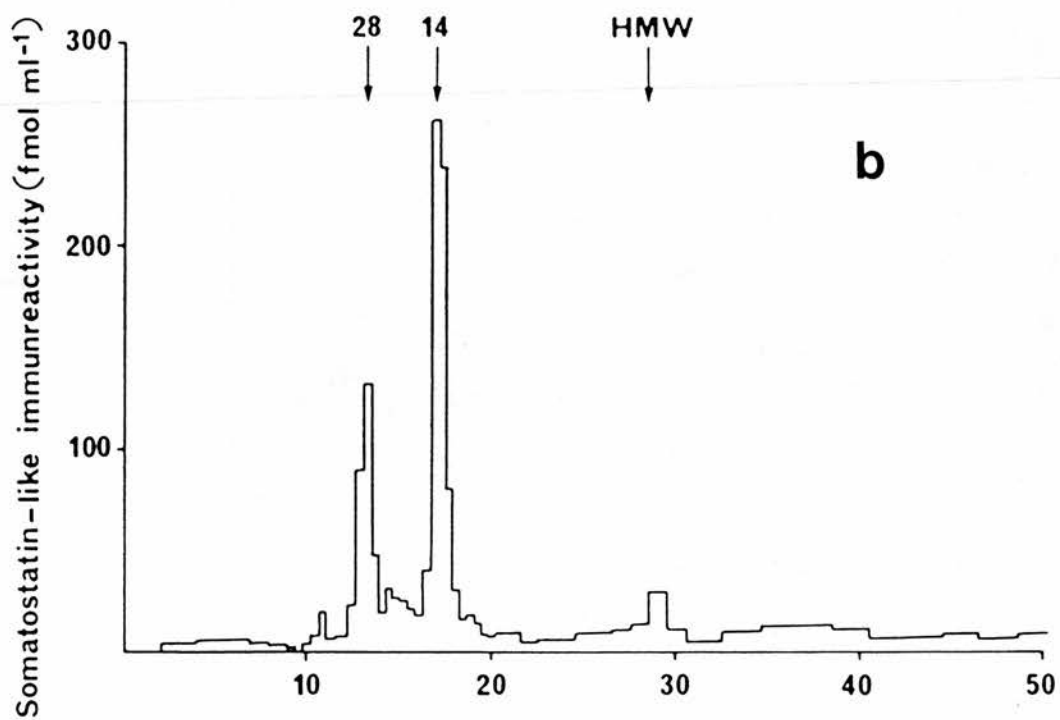
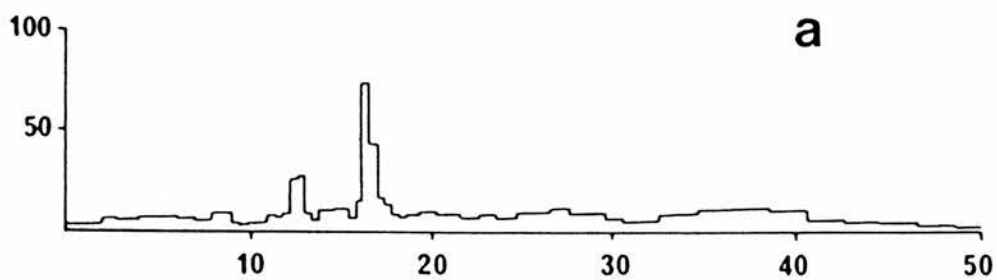




Figure 4.3.4     HPLC analysis of SSLI released from  
isolated median eminences in vitro

Perifusate samples from 60 median eminences were collected for nine minute periods a) before b) during and c) after exposure of tissue to a depolarizing pulse of KCl. HPLC analysis and RIA were performed as described in Chapter 2. Results are expressed as femtomoles SSLI per milliliter of HPLC eluate; the elution positions of synthetic SS28, SS14 and of HMW-SS are indicated.



#### 4.4 DISCUSSION

This study has confirmed that the  $K^+$  evoked release of SSLI from hypothalamic slices and from median eminence tissue in vitro is calcium dependent. Although a small amount of SS28 was released, the predominant form of SSLI released from hypothalamic slices was SS14. The rate of release of SS14 was increased 12 fold after  $K^+$  depolarization whereas the rate of SS28 release was unaltered. In contrast, in the median eminence the rates of release of SS14, SS28 and HMW-SS were increased by 4.3, 5.5 and 1.6 fold respectively.

These results agree with other studies in which total SSLI release in response to a depolarizing pulse of  $K^+$  has been measured (Iversen et al., 1978; Gamse et al., 1980). In a recent study by Charpenet & Patel (1985) the multiple forms of SSLI and of SS28<sub>(1-12)</sub> LI released from the median eminence during  $K^+$  depolarization were examined by gel filtration chromatography. In agreement with this work they found SS14, SS28 and HMW-SS in the stimulated fraction with 22% of the total SSLI present as SS28. Chromatography was not performed on a pre-stimulation sample so the increases in the rate of release of each form could not be established.

In both this study and in the work of Charpenet & Patel (1985) the release of HMW-SS from the median eminence was observed. In Chapter 3, the median eminence has been shown to contain HMW-SS and additionally an immunocytochemical study (Lechan et al., 1983) using an antiserum directed towards residues 39-53 of proSS has shown material containing this sequence in nerve terminals especially in the tubero-infundibular system. The physiological role of proSS

and SS6k in the CNS has not been established but its presence in and release from the median eminence suggests that HMW-SS may act as a neurotransmitter in this tissue.

A number of studies have suggested that SS28 has a greater biological potency than SS14 in inhibiting both GH and insulin secretion in vivo (Brown et al., 1981; Tannenbaum et al., 1982). However, the plasma half-life of SS28 has been reported to be approximately twice that of SS14 (Polonsky et al., 1982) and the results of in vivo studies could, in part, be explained by this finding. Selective actions for SS14 and SS28 have also been observed in studies carried out in vitro. Brazeau et al. (1981) have shown using dispersed anterior pituitary cells, that SS28 is between 3-14 times more potent than SS14 in inhibiting both basal and PGE<sub>2</sub> stimulated GH secretion. Studies on isolated perfused rat pancreatic tissue (Mandarino et al., 1981) have shown that SS28 is ten times more potent than SS14 in the inhibition of insulin secretion, whereas SS14 is ten fold more potent in inhibiting glucagon secretion.

The pancreatic D cell contains predominantly SS14 and does not seem to contribute significantly to circulating levels of SSLI (Taborsky & Ensink, 1984). This finding and the greater in vivo and in vitro potency of SS28 in inhibiting insulin secretion, has led Baskin & Ensink (1984) to propose that SS28 may be preferentially secreted from intestinal mucosal epithelial cells into the circulation to act as a 'hormonal' regulator of insulin secretion from the pancreas. SS14, which is a more potent inhibitor of glucagon than SS28, would be released by pancreatic D cells to act locally inhibiting glucagon secretion.

SS28 is also more potent than SS14 as an inhibitor of growth hormone secretion in vivo and in vitro (Brown et al., 1981; Tannenbaum et al., 1982; Brazeau et al., 1981). Fibres projecting from the rostral periventricular area to the median eminence may preferentially release SS28 into the hypophysial portal vessel circulation to inhibit the release of GH from the pituitary gland. SS14 released by neurones in other hypothalamic nuclei may act as a neuroendocrine regulator.

These findings suggest that in neurones and endocrine cells which secrete somatostatin into the circulation, the post-translational processing of proSS may give rise to SS28. The increased plasma half-life of SS28 means that it is more likely than SS14 to reach its target tissue intact. In neurones where somatostatin has been postulated to act as a neurotransmitter, SS14 is the predominant end product of processing.

Immunohistochemical evidence (Kawano et al., 1982), supports the existence of two somatostatin-containing neuronal systems in rat hypothalamus. One population with cell bodies in the rostral periventricular area sends fibres to the median eminence. The other, with cell bodies in the suprachiasmatic, arcuate and ventromedial nuclei projects to the intra- and/or extra-hypothalamic regions except the median eminence. Additionally, ontogenic studies in rat hypothalamus (Daikoku et al., 1983) show that the somatostatin-containing neurones in the arcuate-ventromedial nuclear region arise on a different day of gestation from those in the rostral periventricular area.

The results of this chapter provide supportive evidence for the existence of two populations of somatostatin-containing neurones in

the hypothalamus. One population, consisting of interneurons in the arcuate, ventromedial and suprachiasmatic nuclei release SS14 as a putative neurotransmitter. The second population with cell bodies in the rostral periventricular area, sends projections to the median eminence and releases both SS14 and SS28 as hormonal regulators of pituitary GH secretion.

## CHAPTER 5

SOMATOSTATIN-LIKE IMMUNOREACTIVITY IN  
ALZHEIMER TYPE DEMENTIA AND DOWN'S SYNDROME

## 5.1 INTRODUCTION

The early symptoms of Alzheimer-type dementia (ATD) are impairment of memory for recent events, dysphasia and depression (Todorov et al., 1975). As the disease advances, all aspects of memory fail progressively, the patients become disorientated, lose motor co-ordination and finally become bedridden, incontinent and unable to feed themselves. Bronchopneumonia is often the final cause of death. Clinically, ATD can usually but not always be distinguished from other dementing conditions such as multi-infarct dementia and Pick's disease. However, a conclusive diagnosis generally depends upon finding characteristic neuropathological changes at post-mortem.

Brains from ATD patients often exhibit moderate to severe atrophy of the temporal, frontal and parietal lobes with wide sulci and narrow flattened gyri as well as an enlargement of the lateral ventricles. These features are distinctive but not necessarily diagnostic and even in severe cases, brain atrophy may not be pronounced. The most widespread neuropathological feature of ATD is the presence of neurofibrillary tangles in neuronal cell bodies in the hippocampus, temporal and frontal cortex. Ultrastructurally, tangles consist of large aggregates of paired helical filaments with an individual filament diameter of 10nm. These are wound together in a double helix with a total diameter of 20nm and a periodicity of 60nm (Kidd, 1963). The second characteristic feature of ATD is the presence of senile plaques, which are abundant in the cortex, hippocampus and amygdala. Plaques are round or ovoid structures of 15-200 $\mu$ m diameter. There are at least three main components of a



typical plaque; abnormal nerve processes, glial processes and amyloid fibrils. The mature plaque is distinguished by a central core of amyloid surrounded by a peripheral rim of neurites, intermixed with glial fibres and glial cell bodies.

Plaques and to a lesser extent tangles, are found in the normal aged human brain, although in much smaller numbers than in ATD. The diagnosis of ATD is based on the presence of an increased number of plaques and tangles in the frontal cortex, temporal cortex and hippocampus at post-mortem compared with age-matched controls.

The first biochemical deficit reported in post-mortem brain ATD was a significant reduction in choline acetyltransferase (ChAT) activity (Bowen et al., 1976; Davies & Maloney, 1976) from hippocampus and temporal cortex. Other cholinergic markers appear to parallel this loss. Synaptosomal choline uptake is reduced in ATD (Rylett et al., 1983) and acetylcholinesterase (AChE) activity is reduced (Davies et al., 1979). There is one report (Richter et al., 1980), that acetylcholine concentrations are reduced and recent work using biopsy specimens (Sims et al., 1983) has shown that the synthesis and release of acetylcholine from tissue prisms is reduced.

The concentration of noradrenaline is reduced in hypothalamus and cerebral cortex in ATD (Adolfsson et al., 1979; Yates et al., 1981; Mann et al., 1982). The activity of dopamine  $\beta$ -hydroxylase, the specific biosynthetic marker enzyme of noradrenergic neurones, is also reduced in cerebral cortex in ATD (Cross et al., 1981).

Most studies agree that there is a reduction in both 5-hydroxytryptamine and its metabolite 5-hydroxyindoleacetic acid (Arai et al., 1984; Gottfries et al., 1983) in cortex and hypothalamus in ATD.

A number of putative neuropeptide transmitters have been measured in post-mortem brain from cases of ATD. The concentrations of VIP, CCK, TRH, LHRH, vasopressin, substance P and neurotensin are generally reported to be unchanged in ATD (Biggins et al., 1983; Ferrier et al., 1983; Perry et al., 1981; Rossor et al., 1980b; Rossor et al., 1980c; Yates et al., 1983a). Of the many peptides examined in ATD, somatostatin is the only one to show a consistent reduction (Davies et al., 1980; Rossor et al., 1980a; Ferrier et al., 1983). The loss of the peptide is greatest in temporal cortex and may spare the frontal lobe in older patients (Rossor et al., 1984). Somatostatin receptors have been reported to be reduced in the temporal cortex in ATD (Beal et al., 1985c). A recent report (De Sousa et al., 1986) has shown that CRH is significantly reduced in cortex in ATD by up to 80%, with a reciprocal increase in CRH binding.

Younger ATD patients in general suffer a clinically more severe disease than older patients, accompanied by greater histopathological changes. Similarly, the biochemical abnormalities appear to be more severe and widespread in younger cases. The reductions in ChAT and AChE activities are much more pronounced in cases of ATD aged under 70 at death (pre-senile ATD) than in cases of ATD aged over 70 at death (senile ATD) (Yates et al., 1983b). In senile ATD the changes appear to be more restricted, with sparing of the frontal lobe when compared to age-matched controls (Rossor et al., 1984). The loss of noradrenergic neurones is more severe in pre-senile ATD than in senile ATD cases (Bondareff et al., 1982; Yates et al., 1983b; Rossor et al., 1984). It has been suggested that the decrease in

SSLI in cortex is greater in pre-senile ATD than in older patients (Crystal & Davies, 1982; Rossor et al., 1984).

An extra copy of chromosome 21 (trisomy 21) is the cause of the disorder known as Down's syndrome (DS). The syndrome is characterized by mental retardation, moderate growth retardation, epicanthal eye folds and some signs of premature ageing. Neuropathological changes typical of ATD were first reported in a patient with DS by Struwe (1929). Further studies (Malamud, 1972; Burger & Vogel, 1973; Ellis et al., 1974; Wisniewski et al., 1979) have shown that the presence of plaques and tangles in DS brain is found in all cases aged over 40. DS patients aged over 40 have also been reported to be demented (Ellis et al., 1974; Wisniewski et al., 1978) although this is a matter of some controversy (Kolata, 1985).

In post-mortem brain, transmitter deficits similar to those observed in ATD have been reported in older cases of DS with ATD pathology. Levels of ChAT and AChE (Yates et al., 1980) noradrenaline (Yates et al., 1983b; Yates et al., 1981) and 5-hydroxytryptamine (Yates et al., 1986) are significantly reduced in older cases of DS. No change in TRH, LHRH or substance P levels is reported in DS (Yates et al., 1983a). However, neurotensin, which is reported to be unchanged in ATD (Biggins et al., 1983; Ferrier et al., 1983), is significantly increased in frontal and temporal cortex and caudate nucleus in DS (Yates et al., 1985).

The similarity between the neuropathological and biochemical changes observed in ATD and older cases of DS suggest that SSLI may be reduced in DS. In order to investigate this, total SSLI was measured in temporal cortex from cases of DS and from age-matched

controls. To establish if the changes in SSLI are more severe in pre-senile ATD than in senile ATD, total SSLI measured in temporal cortex from pre-senile ATD and senile ATD cases was compared to age-matched controls.

Human tissues have been reported to contain similar molecular forms of SSLI to those found in the rat (Wu et al., 1983; Aronin et al., 1983). If the depletion of cortical SSLI seen in ATD was the result of a defect in the biosynthesis or processing of the peptide, a change in the relative proportions of the various forms of SSLI might be observed. The molecular heterogeneity of somatostatin-like peptides was examined in samples of temporal cortex from cases of pre-senile ATD, senile ATD, DS and age-matched control subjects.

## 5.2 METHODS

### 5.2.1 Preparation of tissue samples

Brains were obtained at autopsy from 16 neuropathologically confirmed cases of ATD and 9 cases of DS all with trisomy 21. Seven DS cases aged over 40 had the neuropathological features of ATD with numerous plaques and tangles in the temporal cortex. The remaining two cases of DS were aged under 40 (young Down's) and did not show ATD neuropathology. Control brains were obtained from 20 cases with no clinical signs of CNS abnormality. Seventeen of these brains were examined neuropathologically; some plaques but no tangles were found in seven cases, six aged over 70 and one under 70.

The ATD cases were divided into a group with onset of dementia before 65 and age 70 years or less at death (pre-senile ATD) and a

group aged over 70 years at death (senile ATD). The controls were similarly divided into a group aged 70 years or less (young controls) and an group aged over 70 years at death (old controls). The cases of DS were all less than 70 years at death (Table 5.1). Down's syndrome, young Down's and pre-senile ATD cases were compared with young controls. Cases of senile ATD were compared with the old control group. All cases were hospital in-patients. Cause of death and drugs administered orally within two weeks of death are shown in Tables 5.2 and 5.3.

The cadavers were refrigerated within 4h and the post-mortem carried out within 54h of death. Each brain was divided in the mid-sagittal plane at autopsy and the right hemisphere fixed in 10% formalin for neuropathological examination. The left hemisphere was placed on ice and grey matter from the mid-temporal gyrus dissected, chopped and mixed to ensure homogeneity and stored at  $-70^{\circ}\text{C}$  (Mackay et al., 1978).

For determination of SSLI, frozen temporal cortex (~ 100mg) was homogenized in 1ml of ice-cold 2M acetic acid and a portion of the homogenate removed for protein assay prior to centrifugation for 5min at 8000g. The supernatant was stored at  $-40^{\circ}\text{C}$  until assayed. After recentrifugation (10 min at 8000g), four aliquots within the range of the assay (0.1 - 10 $\mu\text{l}$ ) were dried in a vacuum oven and assayed in duplicate to estimate total SSLI. The remainder of the sample was passed through a Millex GV disposable filtration unit (Millipore, Bedford, MA) before HPLC. ChAT activity was measured by the method of Fonnum (1975) and protein by the method of Peterson (1977) using bovine serum albumin as standard.

TABLE 5.1

## AGE AND POST-MORTEM INTERVAL OF DIAGNOSTIC GROUPS

Diagnostic Group	No. of cases	Age at death range (median)	PM interval range (median)
<u>≤ 70 years</u>			
Young Control	11 (7M 4F)	55-70 (63)	3-26 (19)
Pre-senile ATD	7 (4M 3F)	62-70 (68)	3-36 (23)
Down's syndrome	7 (4M 3F)	53-67 (59)	12-24 (20)
Young Down's syndrome	2 (2F)	27, 37	18, 54
<u>&gt; 70 years</u>			
Old Control	9 (3M 6F)	72-88 (75)	4-47 (21)
Senile ATD	9 (1M 8F)	76-90 (84)	6-30 (15)

M = Male: F = Female

TABLE 5.2

## CAUSE OF DEATH

Cause of death	Young Control	Pre-senile ATD	Down's syndrome	Old Control	Senile ATD
Heart failure	7	1	-	3	2
Broncho-pneumonia	1	6	7	1	4
Aortic aneurysm	2	-	-	-	-
Chronic bronchitis	1	-	-	-	-
Pulmonary embolus	-	-	1	1	1
Peritonitis	-	-	-	1	-
Kidney failure	-	-	-	1	1
Hernia	-	-	-	-	1
Unknown	-	-	1	-	-

TABLE 5.3

## DRUGS GIVEN ORALLY WITHIN TWO WEEKS OF DEATH

Drugs	Young Control	Pre-senile ATD	Down's syndrome	Old Control	Senile ATD
None	-	2	2	-	-
Morphine type Analgesics	7	1	1	1	1
Penothiazines	3	2	1	-	4
Antibiotics	2	2	2	3	2
Diuretics	5	-	-	2	2
Benzodiazepines	1	1	-	1	2
Sympathomimetics (inc. DOPA)	5	1	-	4	1
Thyroxin	-	-	2	1	1
Lithium	-	-	-	1	-
Anti-epileptics	-	1	2	1	-
Steroids	1	1	-	-	-
Not known	1	-	1	2	-



### 5.2.2 Gel filtration chromatography

Gel filtration chromatography was performed on a column of Sephadex G-75 (Superfine, 85cm x 1.6cm) equilibrated with 30% acetic acid and calibrated using cytochrome c (mol. wt. 12,300),  $^{125}\text{I}$ -labelled insulin (mol. wt. 5,700) and substance P (mol. wt. 1,348). The exclusion volume determined by dextran blue 2000 was 38ml. The solvent used for elution was 30% acetic acid and the flow rate was  $5\text{ml h}^{-1}$ . 1.0 – 1.5g of grey matter from the temporal cortex of control, ATD and DS cases was homogenised in 1.5ml of 2M acetic acid. After centrifugation (5 min at 8000g) samples were applied to the column. 1ml fractions were collected and 50 – 200 $\mu\text{l}$  aliquots were dried in a vacuum oven prior to RIA for SSLI as described in section 2.1.

### 5.2.3 HPLC and RIA of cortical extracts

HPLC separation of cortical extracts was carried out as described in Chapter 2. After HPLC, 400 $\mu\text{l}$  fractions were made up to 1ml by the addition of  $\text{H}_2\text{O}/0.2\%$  TFA and 400 $\mu\text{l}$  aliquots of eluate were dried in a vacuum oven prior to RIA. 50–200 $\mu\text{l}$  aliquots of peak fractions were reassayed in duplicate.

## 5.3 RESULTS

### 5.3.1 Total SSLI and ChAT activity

ChAT activity was significantly reduced by over 65% in DS and in both groups of ATD cases compared to age-matched controls (Table 5.4). In contrast, cortical SSLI was significantly reduced ( $p < 0.005$ ) only in pre-senile ATD. In this condition, SSLI was

TABLE 5.4

TOTAL SSLI AND ChAT ACTIVITY IN TEMPORAL CORTEX IN ATD AND DS

Group	No. of cases	SSLI (fmol mg protein <sup>-1</sup> )	ChAT activity (nmol h <sup>-1</sup> mg protein <sup>-1</sup> )
Young control	11	508-923 (602)	2.61-8.16 (3.88)
Pre-senile ATD	7	322-528 (413)*	0.28-1.86 (0.59)**
Down's syndrome	7	396-988 (539)	0.29-2.45 (1.06)**
Young Down's	2	909, 641	533, ND
Old Controls	9	228-797 (457)	2.82-6.07 (4.50)
Senile ATD	9	348-763 (508)	0.31-3.10 (0.89)** (n = 7)

Cortical extracts were prepared as described in section 5.2.1. Estimates of total SSLI were based upon duplicate determinations from four dilutions of each tissue sample: all samples were within the range of the assay and displacement curves were parallel with that of synthetic SSLI. Values are expressed as range (median).

\*p < 0.005; \*\* p < 0.001

Wilcoxon's rank test compared with age-matched controls. ND = not determined.

decreased by 35% compared to age-matched controls. No decrease of SSLI was observed in cases of DS or senile ATD, despite marked losses of ChAT activity. Total SSLI was not significantly correlated with age, post mortem interval, agonal state or ChAT activity in either the control or the ATD groups.

### 5.3.2 Gel filtration chromatography

The heterogeneity of SSLI in human temporal cortex was examined by gel filtration chromatography. Analysis of extracts of temporal cortex from single cases of pre-senile ATD, senile ATD, DS and from one young and one old control subject, revealed four forms of SSLI with apparent molecular weights of 12,000, 7,500, 3,500 and 1,700 (figures 5.3.1 and 5.3.2). HPLC analysis indicated that the 1,700 and 3,500 mol. wt. peaks of SSLI corresponded to synthetic SS14 and SS28 respectively. The 7,500 and 12,000 mol. wt. species eluted from HPLC as a single peak in the same position as the 6,000 and 10,000 mol. wt. forms in the rat (HMW-SS). The 7,500 and 12,000 mol. wt. peaks appeared to be reduced in pre-senile ATD and DS compared to the young control subject. No difference between old control and senile ATD was observed.

### 5.3.3 High performance liquid chromatography of cortical extracts

Samples of temporal cortex from seven cases of pre-senile ATD, six cases of senile ATD, six cases of DS aged over 40, two young Down's cases, seven young and six old controls were subjected to HPLC (Table 5.5, Table 5.6, figures 5.3.3 and 5.3.4). In the pre-senile ATD group, SS14 and HMW-SS were significantly reduced by 44% and 60% respectively (Table 5.5). In DS, levels of HMW-SS were

Figure 5.3.1     Analysis of cortical SSLI from young control, pre-senile ATD and DS cases by gel-filtration chromatography

An extract of 1-1.5g grey matter from young control, pre-senile ATD and DS cases was subjected to Sephadex G-75 chromatography as described in section 5.2.2. Arrows indicate the elution positions of standards used to calibrate the column.

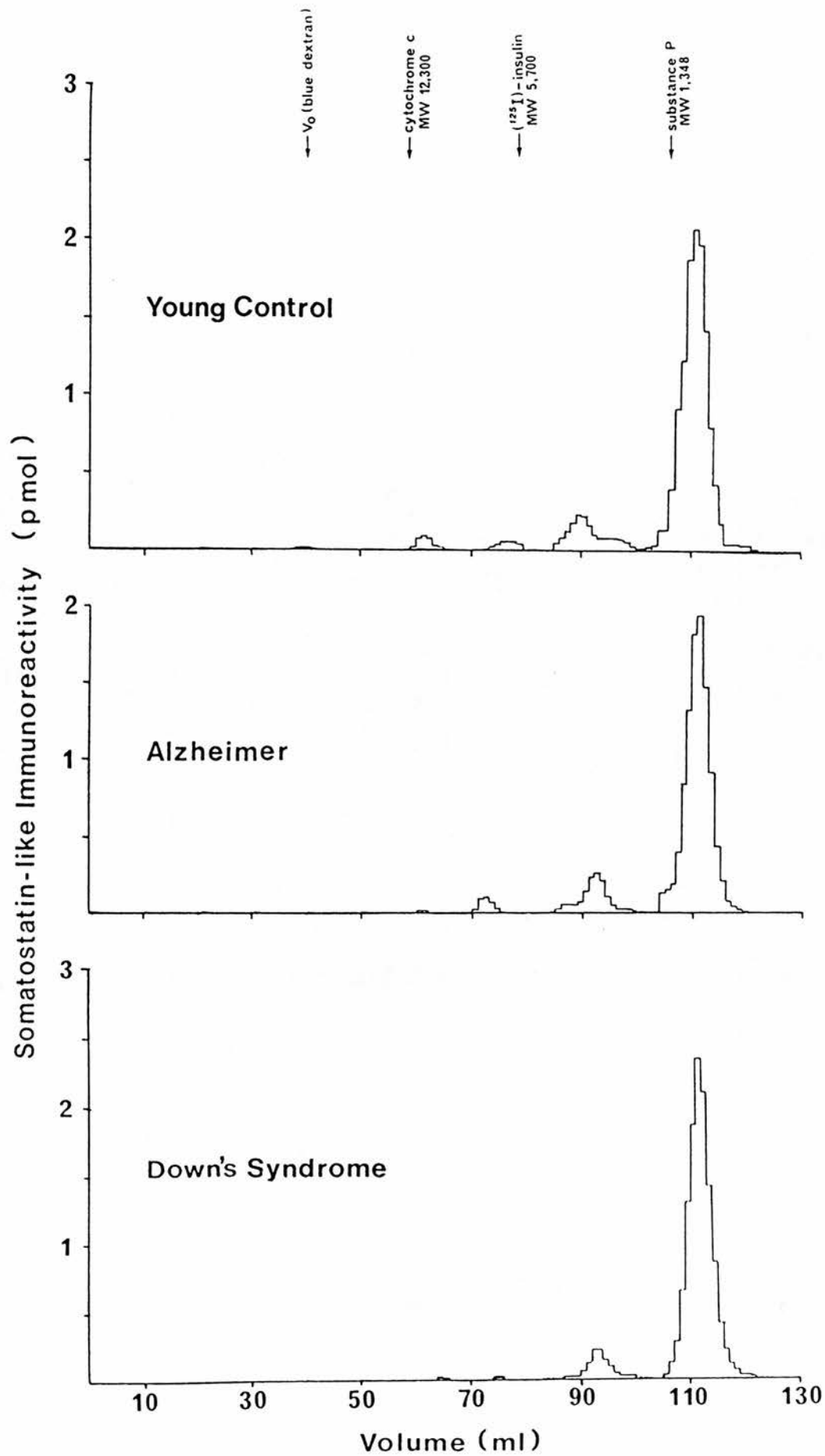


Figure 5.3.2     Analysis of cortical SSLI from old control and senile ATD cases by gel-filtration chromatography

An extract of 1-1.5g grey matter from old control and senile ATD cases was subjected to Sephadex G-75 chromatography as described in section 5.2.2. Arrows indicate the elution positions of standards used to calibrate the column.

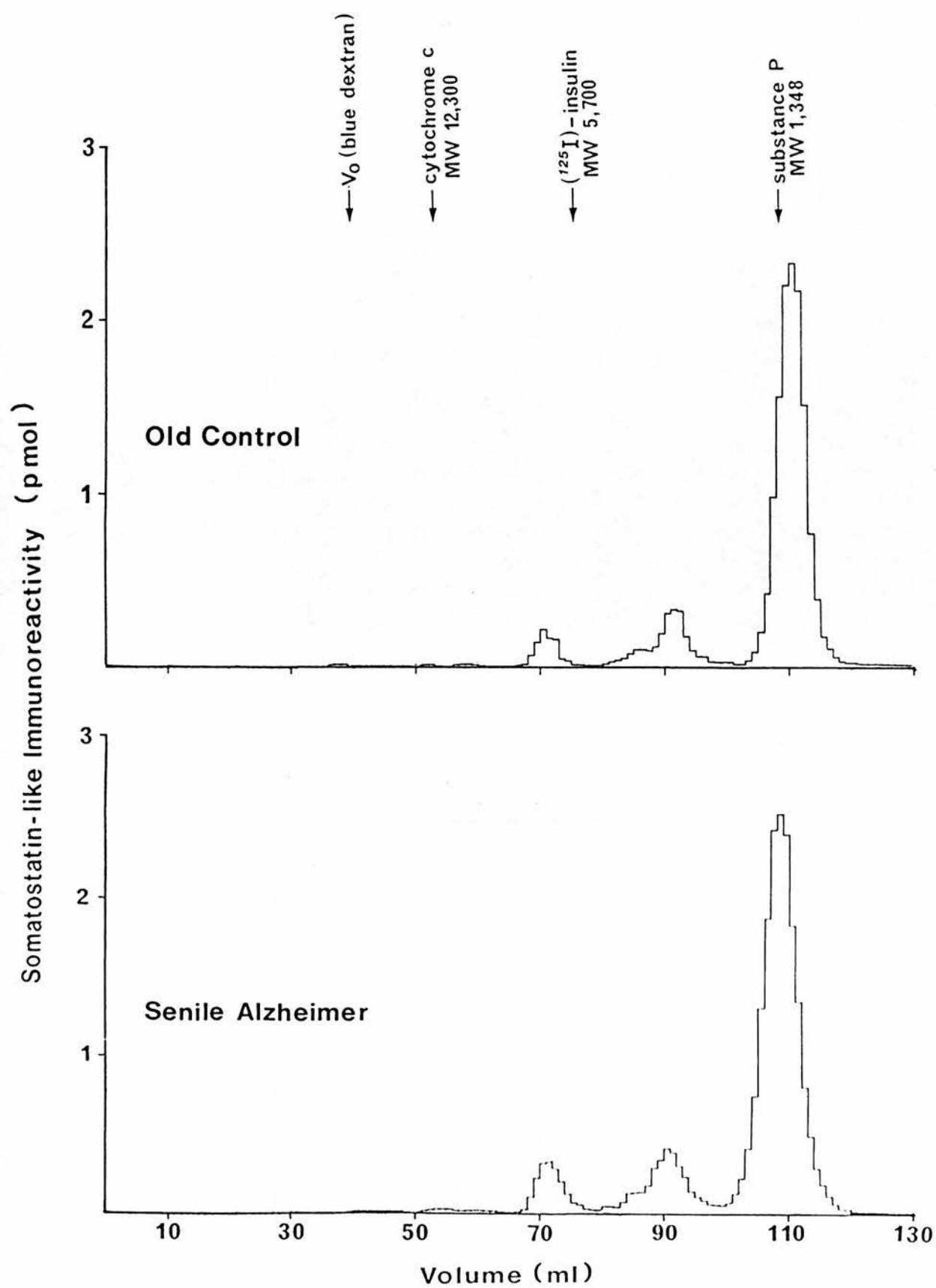


Figure 5.3.3     HPLC analysis of SSLI extracted from the  
temporal cortex of young control,  
pre-senile AD and DS cases

Chromatography and RIA were performed as described in Chapter 2. The elution positions of synthetic SS28, SS14 and of HMW-SS are indicated. Results are expressed as picomoles SSLI per milliliter of HPLC eluate; representative profiles from each condition are shown.



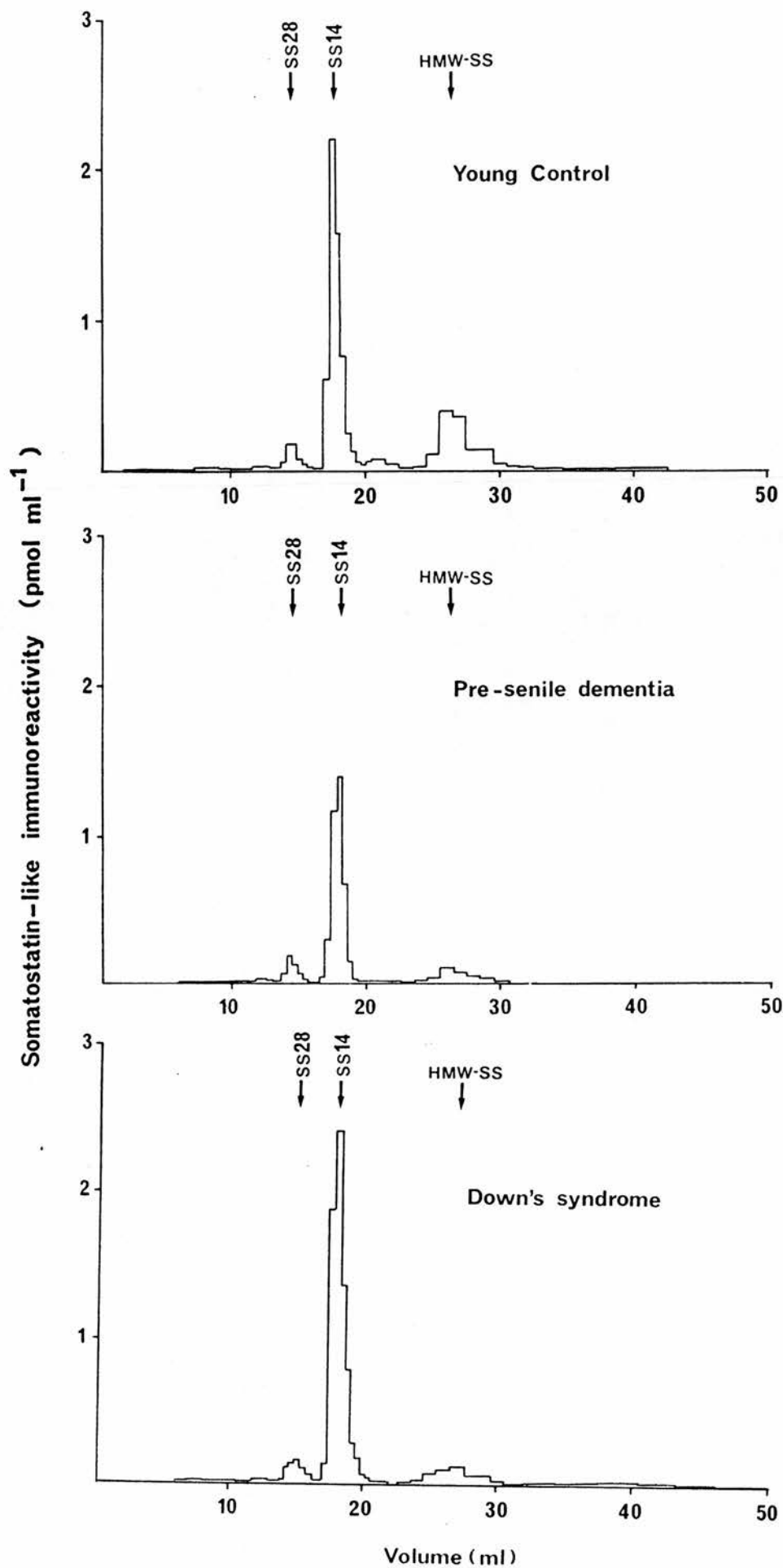


Figure 5.3.4     HPLC analysis of SSLI extracted from the  
temporal cortex of old control and senile  
ATD cases

Chromatography and RIA were performed as described in Chapter 2. The elution positions of synthetic SS28, SS14 and of HMW-SS are indicated. Results are expressed as picomoles SSLI per milliliter of HPLC eluate; representative profiles from each condition are shown.

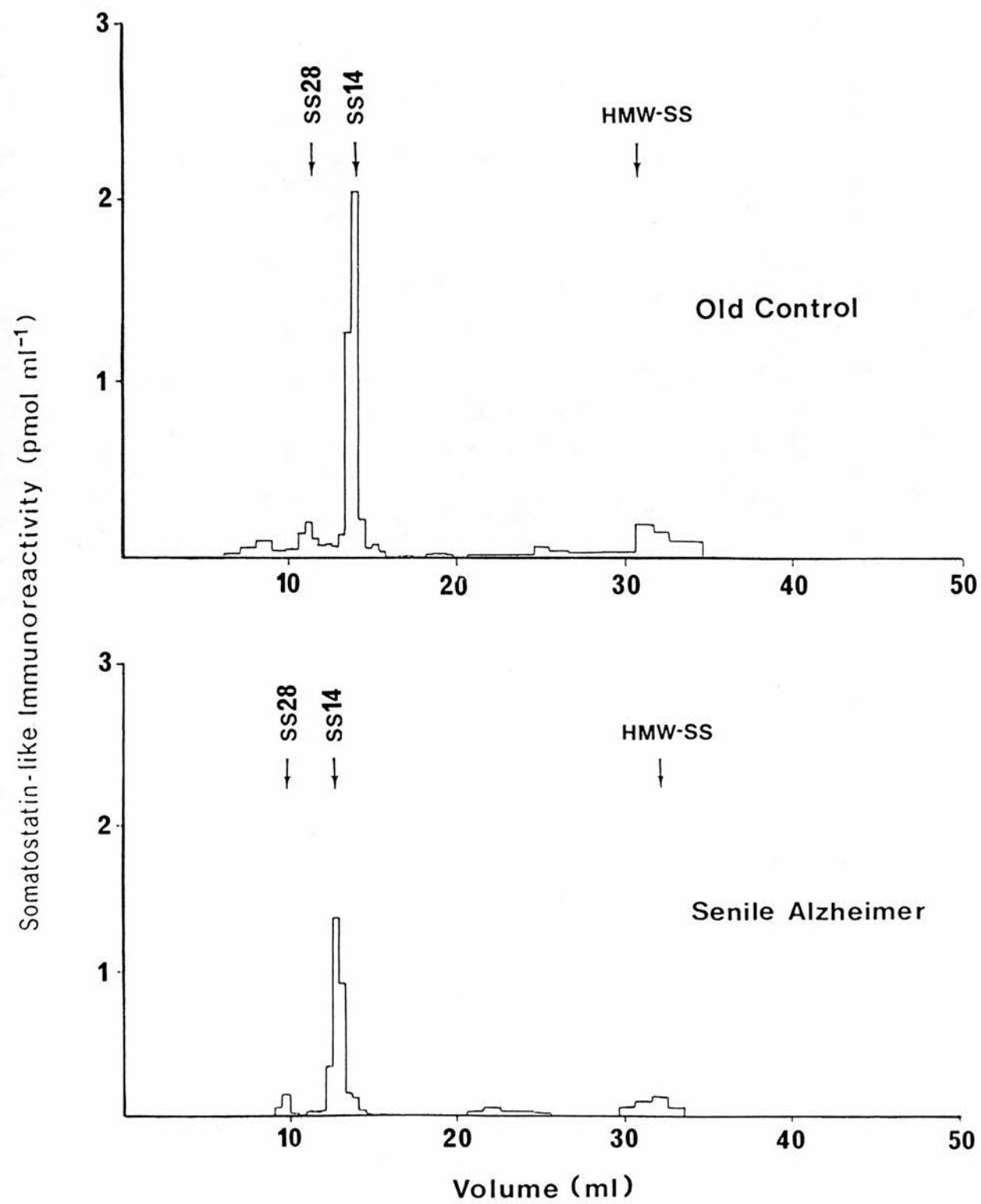


TABLE 5.5

## CONCENTRATION OF MULTIPLE FORMS OF SSLI IN TEMPORAL CORTEX IN ATD AND DS

Group	No. of cases	SS14	SS28	HMW-SS
			(fmol mg protein <sup>-1</sup> )	
Young control	7	126-317 (218)	17-29 (25)	96-183 (142)
Pre-senile ATD	7	98-175 (122)*	9-29 (20)	24-76 (56)**
Down's syndrome	6	102-302 (153)	9-27 (20)	32-103 (60)**
Young Down's	2	309,121	8,11	121,108
Old Control	6	139-339 (227)	10-29 (19)	90-107 (98)+
Senile ATD	6	82-307 (163)	12-33 (15)	30-105 (50)

Cortical extracts from controls and from cases of ATD and DS were subjected to HPLC analysis as described in 5.2.3. Total amounts of SS14, SS28 and HMW-SS were estimated by RIA and are expressed as fmol mg protein<sup>-1</sup>. Values are range (median).

+  $p < 0.02$ ; \*  $p < 0.01$ ; \*\*  $p < 0.005$ .

Wilcoxon's rank test compared with young control values.

TABLE 5.6

## PROPORTION OF MULTIPLE FORMS OF SSLI IN TEMPORAL CORTEX IN ATD AND DS

Group	No. of cases	SS14	SS28 (% of total SSLI)	HMW-SS
Young control	7	41-70 (62)	5-8 (6)	24-51 (32)
Pre-senile ATD	7	54-77 (65)	5-15 (10)**	16-32 (29)*
Down's syndrome	6	53-80 (70)	4-13 (7)	13-34 (25)
Young Down's	2	70,60	2,4	28,36
Old Control	6	53-76 (67)	2-8 (6)	21-38 (28)
Senile ATD	6	57-78 (72)	4-14 (7)	15-37 (22)

Cortical extracts from controls and from cases of ATD and DS were subjected to HPLC analysis as described in section 5.2.3. Total amounts of SS14, SS28 and HMW-SS were estimated by RIA and are expressed as a percentage of total SSLI. Values are range (median).

\*p < 0.05: \*\* p < 0.02.

Wilcoxon's rank test compared with age matched controls.

significantly reduced to 42% of young control values. However, the levels of HMW-SS in the two young Down's cases were similar to control values. HMW-SS was reduced in senile ATD compared with old controls but this change did not reach significance. HMW-SS was significantly reduced by 30% in old controls compared with young controls. No differences were observed between the pre-senile and senile ATD groups. Table 5.6 shows the proportion of each of the molecular forms of SSLI in the extracts. The proportion of HMW-SS was significantly reduced in pre-senile ATD ( $p < 0.05$ ) and the proportion of SS28 significantly increased. The proportion of HMW-SS in DS was reduced but this reduction did not reach significance.

#### 5.4 DISCUSSION

This study has shown that total SSLI was reduced in cases of pre-senile ATD compared with age-matched controls. The concentration of SSLI was unaltered in cases of DS aged over 40 with the neurochemical changes associated with ATD. In senile ATD cases, no change in total SSLI was observed. When the multiple forms of SSLI were examined by HPLC, SS14 and HMW-SS were significantly reduced in cases of pre-senile ATD. The relative proportion of HMW-SS was significantly reduced in this condition. Although SS14 was not decreased in DS, HMW-SS was significantly reduced. No differences between the multiple forms of SSLI present in senile ATD and old controls were seen.

The concentration of HMW-SS was significantly reduced in old controls compared with young controls. This suggests that the

concentration of HMW-SS may be age-dependent. However, there was not a significant correlation between age and HMW-SS in either the control or ATD groups.

Although it is difficult to draw any firm conclusions from the measurement of SSLI in the two cases of young Down's, the concentration of HMW-SS in both cases lay above the range seen in older DS cases and pre-senile ATD. This suggests that the reduction in HMW-SS observed in cases of DS aged over 40 is related to the presence of ATD pathology and is not a consequence of DS per se.

The concentration of SSLI observed in temporal cortex from control cases was similar to published estimates (Aronin et al., 1983; Ferrier et al., 1983; Rossor et al., 1984) and the reduction in total SSLI in the temporal cortex in ATD was confirmed (Davies et al., 1980; Rossor et al., 1980a; Ferrier et al., 1983). In this study, decreased SSLI levels were observed only in pre-senile cases and not, as reported by Rossor et al. (1984), in older cases of ATD. In addition to more severe reductions in SSLI, changes in cholinergic and noradrenergic markers are more pronounced and more widespread throughout the brain in younger cases of ATD (Rossor et al., 1984; Yates et al., 1983b). This finding provides further evidence that cases of pre-senile ATD have a clinically and neuropathologically more severe form of dementia and should be considered separately from cases of senile ATD. The neurochemical and neuropathological changes observed in senile ATD are not much greater than changes associated with normal ageing processes (Mann et al., 1984).

This study has shown that the major constituent of SSLI in human

cerebral cortex is SS14, which is probably the principal form of SSLI stored in and released from cortical nerve terminals. The 12,000 and 7,500 mol. wt. forms of somatostatin are likely to be the prohormone and an intermediate in the biosynthesis of SS14. If the loss of SSLI observed in temporal cortex in pre-senile ATD was due solely to death of somatostatin interneurons, a parallel loss of all forms of SSLI would be expected. However, in pre-senile ATD the proportion of SSLI present as HMW-SS was significantly less than in control cases and the proportion of SS28 was significantly elevated (Table 5.6). In DS, the proportion of SSLI present as HMW-SS was reduced, but this change did not reach significance.

In a tissue such as human temporal cortex which produces predominantly SS14, it is assumed that the prohormone is cleaved via the 6,000-7,500 intermediate and SS28 to SS14, with the ribosomal synthesis of proSS matched by processing and release of SS14. A reduction in the rate of synthesis of the prohormone combined with the rate of proteolytic processing remaining the same or increasing, would explain the changes in the proportions of the molecular forms of SSLI seen in both pre-senile ATD and DS.

Various factors may contribute to a reduction in the rate of synthesis of proSS in affected regions of ATD brain. The reduction may be the consequence of an alteration in brain RNA or DNA levels, or may result from changes in transcription or translation.

Robbins et al. (1983), have reported that fibroblast cell lines from patients with ATD show significantly reduced survival after X-irradiation compared with control cell lines. Li & Kaminskas (1985) have shown a deficient repair of DNA after damage induced by N-methyl-N'-nitro-N-nitrosoguanidine in fibroblasts from ATD



patients. Impaired repair of DNA is also observed in DS (Lambert et al., 1976; Countryman et al., 1977) suggesting that DS and ATD may share a common abnormality in the repair or metabolism of DNA. Accumulation of unrepaired DNA strand breaks occurs with ageing in the mouse brain (Chetsanga et al., 1979). A similar phenomenon may occur in ageing human brain leading to decreased protein synthesis.

A significant reduction in levels of both ribosomal RNA (rRNA) and messenger RNA (mRNA) has been observed in affected ATD cortex (Sajdel-Sulkowska & Marotta, 1984). Recently, a reduction in the level of SS-mRNA to 70% of control values has been reported (Taylor et al., 1986). The activity of alkaline ribonuclease, an RNA degrading enzyme found in the soluble fraction of brain homogenates is increased by over 80% in pathologically affected areas of ATD brain (Sajdel-Sulkowska & Marotta, 1984). This increased ribonuclease activity has been attributed to a decrease in the concentration of an endogenous inhibitor.

Decreased SS-mRNA levels due to increased ribonuclease activity or reduced levels of DNA encoding proSS due to deficient repair would both lead to reduced synthesis of proSS. Alterations in the proportions of molecular forms of somatostatin similar to those observed in pre-senile ATD and DS would be expected to result from these changes.

Changes in protein concentration and protein synthesis have also been shown in ATD. In post-mortem brain from cases of ATD, the concentration of soluble protein in frontal cortex was reduced (Smith & Bowen, 1976), with losses of soluble proteins of molecular weight 55,000, 30,000 and 28,000 in the temporal cortex (Borthwick et al., 1985a). Losses of soluble proteins of these molecular

weights have also been observed in cases of DS with ATD pathology (Borthwick et al., 1985b). In a study using positron emission tomography with  $^{11}\text{C}$ -L-methionine as tracer, Bustany et al. (1983) showed a 65% decrease in the rate of protein synthesis in severely demented patients.

Neurofibrillary tangles are common in somatostatin containing neurons (Roberts et al., 1985) and SSLI has also been observed in senile plaques (Armstrong et al., 1984; Morrison et al., 1985). In affected areas of ATD cortex, it is possible that synthesis of mRNA encoding plaque and tangle proteins or structural protein may proceed at the expense of the synthesis of mRNA encoding secreted protein including SS-mRNA. A reduction in SS-mRNA would lead to decreased levels of HMW-SS after translation.

It remains to be established whether the changes in cortical SSLI reported in this chapter are symptomatic of the neuropathological processes involved in plaque and tangle formation or reflect a more generalised disturbance of brain protein synthesis in Alzheimer-type dementia.

## CHAPTER 6

### EFFECT OF ELECTROLYTIC SEPTAL LESIONS ON MULTIPLE FORMS OF SOMATOSTATIN IN THE RAT HIPPOCAMPUS

## 6.1 INTRODUCTION

In Alzheimer-type dementia, the hippocampus and related regions contain numerous neurofibrillary tangles and senile plaques (Bowen et al., 1976; Davies & Maloney, 1976) and are among the brain areas most obviously implicated in the pathology of the disorder. However, it is uncertain to what degree these pathological changes contribute to the memory impairment characteristic of ATD. In the hippocampus and cerebral cortex, a significant reduction in ChAT activity is observed (Bowen et al., 1976; Davies & Maloney, 1976). Measures of other presynaptic markers for cholinergic neurones indicate losses similar to that of ChAT suggesting that the loss is not enzyme specific but reflects a loss of cholinergic neurones.

Most cholinergic neurones projecting to the cerebral cortex and hippocampus are located in the basal forebrain (Fibiger, 1982). The cholinergic input to the hippocampus is derived from cell bodies in the medial septal nucleus and the vertical limb of the diagonal band of Broca. The tracts from the diagonal band pass through the lateral septum and continue, along with the tracts from the medial septal nucleus, through the fornix to the hippocampi. Lesioning of the septum or fornix causes a massive reduction in ChAT activity in the hippocampus (Lewis & Shute 1967; Lewis et al., 1967) This reduction is similar to that observed in ATD, suggesting that lesioning of the septum may be a good model for the study of ATD.

The data demonstrating a severe cholinergic deficit in ATD has led several groups to propose that the primary dysfunction in the disorder is cholinergic (Coyle et al., 1983; Terry & Katzman, 1983; McGeer, 1984). If the primary dysfunction in ATD is

cholinergic, then the changes in other neurotransmitters including somatostatin, that are observed in ATD, must be secondary to the cholinergic deficit. To establish if the changes in total SSLI or in the molecular forms of SSLI observed in ATD and DS (Chapter 5) are secondary to the reduction in ChAT activity, the molecular heterogeneity of SSLI was examined in the hippocampi of control rats and in rats with an electrolytic lesion of the septum.

## 6.2 METHODS

### 6.2.1 Generation of lesions

The animals used were male Han-Wistar rats (body wt. 200-240g) which were maintained under controlled lighting (lights on from 0500-1900h) and temperature (22°C) and allowed free access to food (diet 41B: Oxoid, Basingstoke, Hants) and tap water. Following surgical procedures, the diet was supplemented for ten days with brown bread and milk and Aureomycin (800mg l<sup>-1</sup>: Cyamid GB, Gosport, Hants.) was included in the drinking water. The animals were anaesthetized by intraperitoneal injection of 1ml of 2,2,2-tribromoethanol solution (2ml 5.7M tribromoethanol in 3-methylbutan-1-ol (isoamylalcohol) plus 8ml absolute alcohol and 90ml 0.9% saline) per 100g body weight. Electrolytic or sham lesions were placed in the septum using a platinum electrode which was glass-coated to within 0.5mm of the tip. A current of 2.0mA was applied for 30 seconds using a D.C. lesion maker (model No. DC-LM5; Grass Instruments, Quincy, MA) after the electrode tip had been placed 0.6mm rostral from bregma and 5.9mm ventral from the skull surface. In sham-operated animals, the electrode was lowered

only 3.9mm and current was not applied. Fourteen days later, the rats were killed by decapitation and the left and right hippocampi dissected out and finely chopped with a scalpel blade. The correct placement of the lesion was confirmed by microscopic examination of 40 $\mu$ m serial coronal sections through the plane of the septum which were stained with toluidine blue. A control and a lesioned septum are shown in figure 6.2.1.

#### 6.2.2 Preparation of tissue

10mg of tissue was taken for the estimation of protein and ChAT activity. The remainder of the sample was homogenised in 1ml of ice-cold 2M acetic acid using an all-glass homogeniser and centrifuged for 10 minutes at 8,000g. A 50 $\mu$ l aliquot was taken for the estimation of total SSLI and the remainder of the sample was passed through a Millex GV disposable filtration unit (Millipore, Bedford, MA) before HPLC.

#### 6.2.3 Estimation of protein and ChAT activity

Protein was determined by the method of Petersen (1977) using bovine serum albumin as standard. 1mg of tissue was assayed in duplicate. 200 $\mu$ g aliquots of tissue were assayed in duplicate to determine ChAT activity (Fonum, 1975).

#### 6.2.4 HPLC and RIA

HPLC and RIA for somatostatin were carried out as described in Chapter 2. The 400 $\mu$ l fractions were made up to 1ml by the addition of H<sub>2</sub>O/0.2%TFA and 200 $\mu$ l aliquots of HPLC eluate were dried in a vacuum oven and assayed to determine the multiple forms of SSLI

a



b

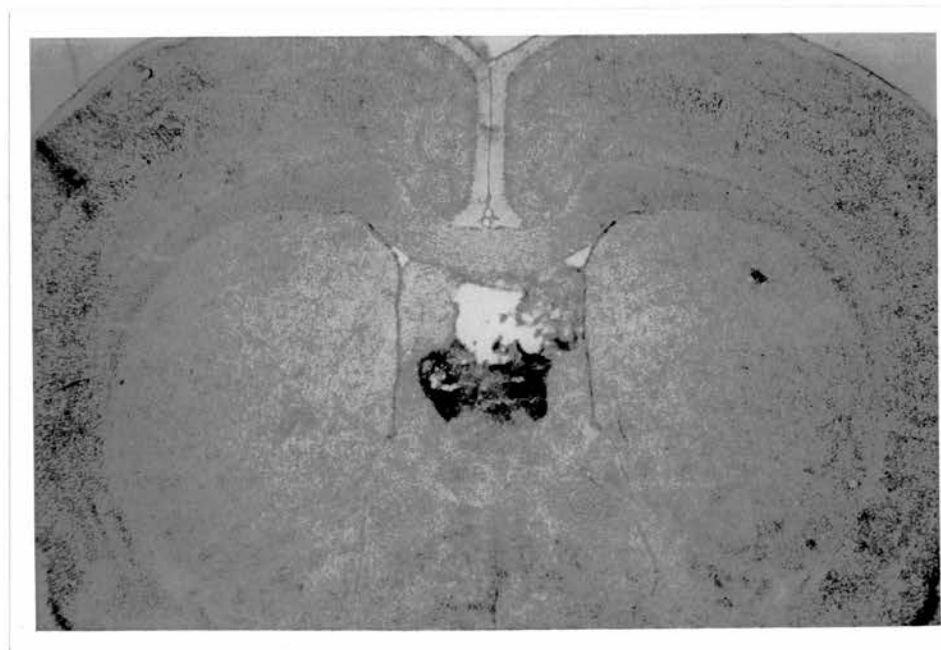


Figure 6.2.1 Coronal sections of rat brain showing the effect of septal lesions

Septal lesions were performed as described in section 6.2.1. The viability of the lesion was confirmed by examination of 40 $\mu$ m coronal sections at the level of the septum which were stained with toluidine blue

a) sham lesion    b) septal lesion

present. 20-100 $\mu$ l aliquots of peak fractions were reassayed in duplicate to quantitate SSLI present. 1-10 $\mu$ l aliquots of unfiltered supernatant were assayed in duplicate to determine total SSLI.

### 6.3 RESULTS

An electrolytic lesion of the septum significantly reduced the ChAT activity of the hippocampus to 11% of control values (Table 6.1). HPLC analysis showed that the predominant form of SSLI in the rat hippocampus was SS14 accounting for 60-70% of the total SSLI. 5% of the total SSLI was SS28 with the remainder present as HMW-SS (Table 6.1). The total SSLI content of the hippocampus was unaltered and the proportions of the multiple molecular forms of SSLI were not significantly altered in lesioned animals compared to sham operated controls. Representative chromatograms from a sham-operated and a lesioned animal are shown in figure 6.3.1.

### 6.4 DISCUSSION

Electrolytic lesions of the rat septum significantly reduced ChAT activity in the hippocampus by approximately 89%. No change in the total SSLI content of the tissue or in the proportions of the multiple forms of SSLI present were observed.

The proportions of the multiple forms of SSLI in the hippocampus resemble those observed in the human temporal cortex from control cases (Chapter 5) and from rat hypothalamus (Chapter 3). The predominant form of the peptide was SS14 with only small amounts of



Figure 6.3.1     HPLC analysis of SSLI extracted from the  
hippocampi of control and lesioned animals

HPLC and RIA were performed as described in Chapter 2. The elution positions of synthetic SS28, SS14 and of HMW-SS are indicated; representative profiles from control and lesioned animals are shown.

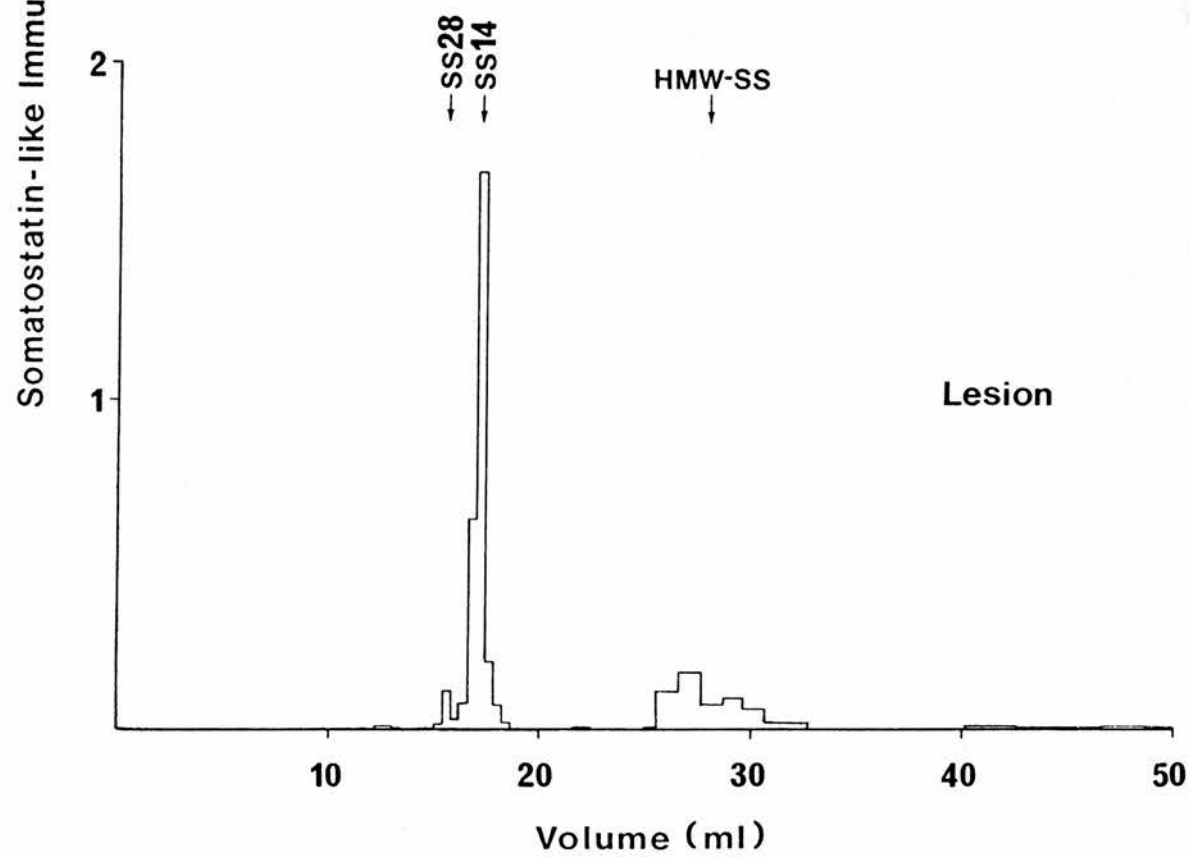
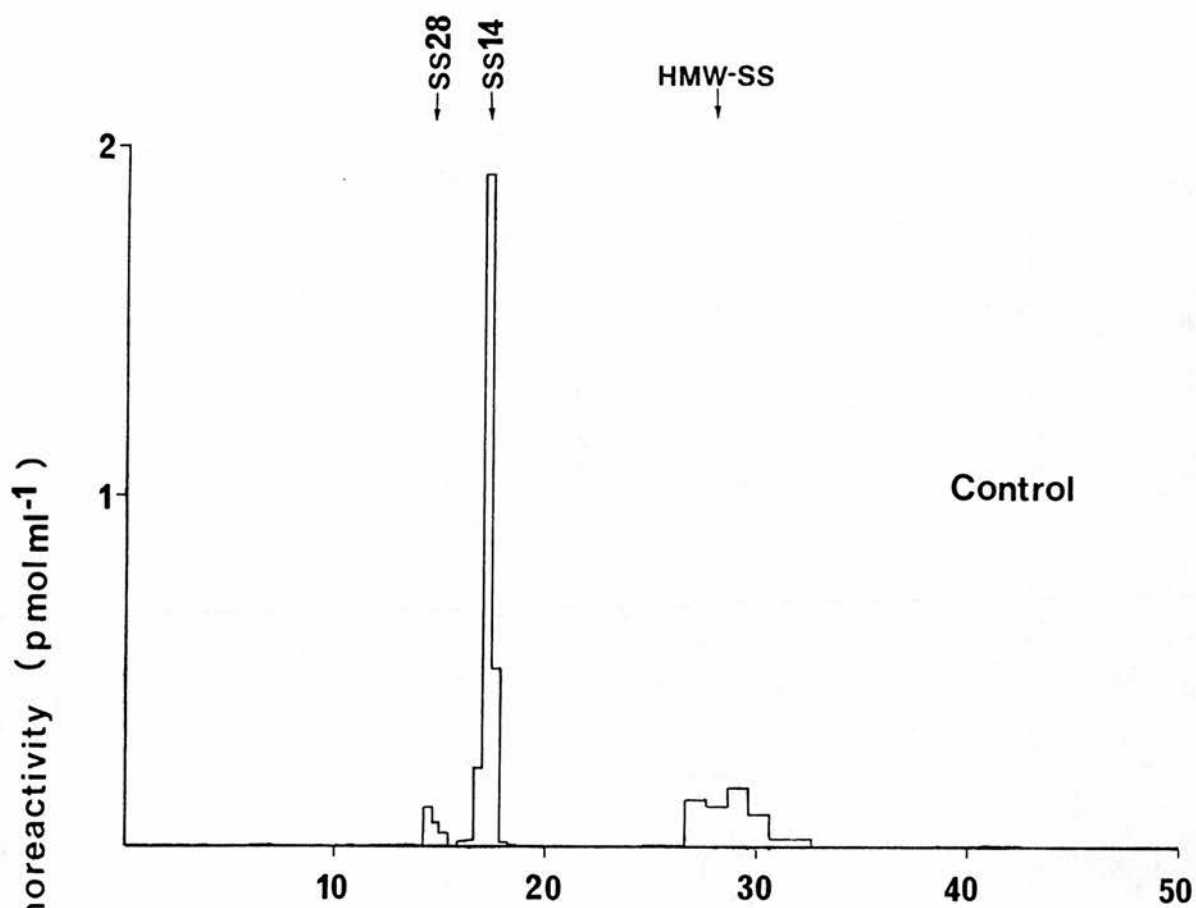


TABLE 6.1

CHAT ACTIVITY AND PROPORTIONS OF MULTIPLE FORMS OF SSLI  
IN RAT HIPPOCAMPUS AFTER SEPTAL LESION

	ChAT activity (nmolh <sup>-1</sup> mg protein <sup>-1</sup> )	Total SSLI (pmol mg protein <sup>-1</sup> )	Proportions of molecular forms (%) SS14	SS28	HMW-SS
Sham lesion (n = 6)	62 ± 4	1.14 ± 0.36	67.0 ± 15.8	4.2 ± 3.0	28.8 ± 15.0
Septal lesion (n = 6)	7 ± 5*	1.45 ± 0.45	63.6 ± 13.5	4.9 ± 1.2	31.5 ± 13.7

Tissue extracts were prepared as described in section 6.2.2. Estimates of total SSLI were based upon duplicate determinations from four dilutions of each sample; all samples were within the range of the assay and displacement curves were parallel with that of synthetic SS14. Tissue extracts were subjected to HPLC analysis as described in section 6.2.4. Total amounts of SS14, SS28 and HMW-SS are expressed as a percentage of total SSLI. Values are mean ± SD. The number of determinations is shown in parenthesis.

\* p > 0.001 Student's t-test

SS28 present, suggesting that SS14 is the form of the SSLI important in neurotransmission in hippocampal interneurons.

These results are in agreement with those of McKinney et al. (1982) who showed that transection of the fornix caused a 63% reduction in the activity of ChAT without altering the concentration of total SSLI in the hippocampus. SSLI levels were also unaltered in the hippocampus after lesions of the brainstem, olfactory bulb, amygdala, striatum and periventricular hypothalamus (Beal et al., 1985a).

Kainic acid lesions of the dorsal hippocampus reduced the content of SSLI in the tissue by 69% without altering the levels of ChAT activity (McKinney et al., 1982), suggesting that SSLI is contained within neurons intrinsic to the hippocampal formation.

The results of Chapter 5 have shown that the changes in ChAT activity seen in both ATD and DS were accompanied by a reduction in total SSLI in cases of pre-senile ATD only. Total SSLI was not reduced in cases of DS aged over 40 with the neuropathological features of ATD. Electrolytic lesions of the rat septum produced a significant reduction in ChAT activity in the hippocampus similar to that observed in cases of ATD and DS but did not alter total SSLI.

In both pre-senile ATD and DS, a reduction in HMW-SS was observed. If the reduction in HMW-SS in these conditions was the result of a loss of cholinergic input which regulated the post-translational processing of proSS, reductions in HMW-SS in the hippocampus should be observed after septal lesioning. However, in lesioned animals, no change in the proportions of molecular forms of SSLI were observed.

These results suggest that the synthesis and processing of

somatostatin in hippocampal interneurons is not influenced by the ascending cholinergic input and provide evidence that the changes in SSLI observed in ATD and DS are not secondary to the cholinergic deficit.

## CHAPTER 7

### SUMMARY AND CONCLUSIONS

### 7.1 Multiple forms of somatostatin in rat brain

The presence of multiple forms of SSLI in the rat hypothalamus and amygdala was confirmed using a sensitive radioimmunoassay in conjunction with gel filtration chromatography and HPLC (Chapter 3). Gel filtration chromatography of hypothalamic extracts revealed the presence of four forms of SSLI with molecular weights of 1500, 3000, 6000 and 10,000. Subsequent analysis by HPLC indicated that the 1500 and 3000 mol. wt. forms corresponded to SS14 and SS28 respectively. The 6000 and 10,000 mol. wt. forms eluted together on HPLC as a composite peak of HMW-SS. The proportions of SS14 (63%) SS28 (12%) and HMW-SS (25%) present in the hypothalamus were similar to those measured in the amygdala (59, 9 and 32% respectively) and hippocampus (64, 4, and 29%). In contrast, the median eminence contained a greater proportion of SS28 than the other tissues, accounting for 34% of the total immunoreactivity. SS25 was not detected in tissue extracts.

The results of Chapter 4 confirmed that the  $K^+$ -evoked release of SSLI from hypothalamic slices and from the isolated median eminence was  $Ca^{2+}$  dependent. The predominant form of SSLI released from hypothalamic slices was SS14, accounting for 76% of the total immunoreactivity after  $K^+$  depolarization.  $K^+$  depolarization increased by 12 fold the rate of release of SS14 but did not significantly influence the rate of release of SS28. In contrast, perifusates of median eminence tissue contained a strikingly different pattern of SSLI from perifusates of the whole hypothalamus: in addition to SS14 (58%) significant amounts of SS28 (25%) and HMW-SS (17%) were present. A depolarizing stimulus increased the rates of release of the three forms by 4.3, 5.5 and

1.6 fold respectively.

These studies indicate that proSS is subject to tissue specific post-translational processing. Such tissue-specific processing could be accounted for by the existence of two or more somatostatin-producing cell types: cells which produce SS14 (Type I), cells which produce SS28 (Type II) and possibly cells which produce both SS14 and SS28 (Type III). Tissues such as the median eminence which contain SS14 and SS28 in comparable amounts may either be innervated by both Type I and Type II neurones or by Type III neurones only.

A number of experiments could be carried out to attempt to identify specific somatostatin-producing cell types. Using specific antisera to SS14, SS28 and SS28<sub>(1-12)</sub> it may be possible to identify immunohistochemically Type I and Type II neurones and to map their distribution. Additionally, it may be possible to establish whether the median eminence contains both Type I and Type II neurones or is innervated by Type III neurones.

Type II cells should stain immunohistochemically for SS28 in both perikarya and nerve terminals but should not stain for SS28<sub>(1-12)</sub>. Type I neurones should contain SS14 and SS28<sub>(1-12)</sub> in nerve terminals but will stain in the cell body region for SS28. This will make such studies difficult to interpret since identification of cell type could only be carried out by examining the staining in the nerve terminals.

At present the pathways by which proSS is cleaved into SS14 and SS28 are not known. Different biosynthetic pathways may operate in Type I, II and III cells and the activities of processing enzymes present in the three cell types may differ. The pathways of



biosynthesis could be studied by incorporation of radiolabelled amino acids, isolation of products by immunoprecipitation using specific antisera and analysis by HPLC. These studies should help to resolve the biosynthetic pathways for SS14 and SS28 in different cell types. Additionally, such studies may establish whether SS14 can be produced directly from proSS and what role SS6k plays in the biosynthesis of SS14 and SS28.

Some work has been carried out on the characterization of the SS28 convertase which cleaves SS28 into SS14 at a pair of basic amino acids (Gluschankof et al., 1984). However, little is known about the enzyme which cleaves at the single arginyl residue to produce SS28. The further identification and characterization of somatostatin processing enzymes may help in understanding the biosynthesis of SS14 and SS28. Additionally, it may provide an alternative method of identifying different somatostatin cell types. Specific antisera could be used to map the distribution of somatostatin processing enzymes. A Type II cell would not be expected to stain with antisera raised against the SS28 convertase.

The predominant form of SSLI in most parts of the CNS is SS14, where it has been postulated to act as a neurotransmitter. SS14 is also released from D cells in the pancreas where it acts as a paracrine factor inhibiting the release of glucagon and possibly insulin. SS28 is the predominant form of SSLI in D cells in the mucosal layer of the ileum and stomach where it is released into the circulation. SS28 may be released into the bloodstream in preference to SS14 because it has an increased plasma half-life compared to SS14. This means it is more likely to reach its target tissue intact.

Reports that SS28 has a greater in vitro and in vivo potency in inhibiting GH secretion (Tannenbaum et al., 1982; Brown et al., 1981; Brazeau et al., 1981) suggest that SS28 may be the physiological inhibitor of GH secretion from the pituitary gland. This is supported by distribution data (Chapter 3) which shows that the median eminence contains a greater proportion of SS28 than the rest of the hypothalamus and by release studies (Chapter 4) which show that the rate of release of SS28 is increased after  $K^+$  depolarization in the median eminence but not in the rest of the hypothalamus. However, the median eminence releases both SS14 and SS28: the rate of release of both peptides is increased by ~ 5 fold after  $K^+$  depolarization.

SS28 may be released by the median eminence into hypophyseal portal vessel blood because it is more stable in plasma than SS14 and, therefore, more likely to reach the pituitary gland intact. It is less clear why SS14 is also released by the median eminence. SS14 and SS28 may act synergistically to inhibit the release of GH from the pituitary gland. Alternatively SS14 may perform a different function from SS28. At present, little is known concerning the relative potencies of SS14 and SS28 in regulating the release of other hypothalamic and pituitary peptides. However, it is possible that SS14 and SS28 act on different targets.

The paradoxical elevation of GH release after intraventricular somatostatin has led Lumpkin et al. (1981) to propose that somatostatin regulates its own release via an ultrashort-loop feedback. SS28 may act to inhibit the release of GH, whereas the shorter acting SS14 may feedback onto receptors in the median eminence to inhibit somatostatin release.

If the median eminence was innervated by Type III neurones, there would not be a mechanism by which the release of SS14 and SS28 could be regulated independently. However, if the median eminence were innervated by both Type I and Type II neurones, the release of SS14 and SS28 could be regulated independently. Regulators of somatostatin release in vivo might then act on only one type of neurone, releasing or inhibiting the release of SS14 or of SS28.

Lesion studies (Crowley & Terry, 1980) support the hypothesis that the median eminence is innervated by two populations of somatostatin-containing neurones. Lesions in the periventricular hypothalamus reduce the SSLI content of the median eminence by 60-70%. Additionally, SSLI in the median eminence is reduced by ~ 30% after lesions in the medial-based amygdaloid nuclei. Combined periventricular nucleus and medial amygdala lesions caused a 91% decrease in the level of SSLI in the median eminence. This suggests that a second somatostatinergic pathway to the median eminence exists, projecting from the amygdala via the stria terminalis.

There is evidence that these two somatostatinergic pathways may perform different neuroendocrine functions. Lesions of the amygdala, which have no effect on GH levels, significantly increase plasma TSH levels, while lesions of the periventricular hypothalamus cause a small reduction in TSH levels which may be due to damage to TRH-containing neurones passing through the periventricular area (Terry & Crowley, 1986).

The somatostatin fibres which project from the amygdala, may be of Type I, whereas the projection from the periventricular hypothalamus may be of Type II. Type II neurones which release

SS28, would inhibit the secretion of GH from the pituitary gland whereas SS14 released from Type I neurones could be involved in the inhibition of TSH secretion. Alternatively, since SS14 is capable of inhibiting TSH secretion indirectly by inhibiting TRH release at the hypothalamic level (Hirooka et al., 1978), SS14 released from Type I neurones may act locally to inhibit the release of TRH from the median eminence.

Further evidence for the existence of two somatostatin-containing pathways projecting to the median eminence could be obtained by examination of the forms of SSLI contained in or released from the median eminence of rats after lesions of the amygdala or anterior periventricular area. The effects of selective electrical stimulation of the amygdala or anterior periventricular area upon the release of SS14 and SS28 could also be examined. Further experiments measuring the release of pituitary hormones after selective lesions or electrical stimulation may establish separate functions for SS14 and SS28.

## 7.2 Multiple forms of somatostatin in human brain

Four molecular forms of SSLI were observed in human temporal cortex; SS14, SS28 and 7,500 and 12,000 mol. wt. forms. The 7,500 and 12,000 mol. wt. forms eluted as a composite peak of HMW-SS after HPLC (Chapter 5). Temporal cortex from both ATD and DS cases contained the same molecular forms as controls. SS14 and HMW-SS were depleted by 44% and 60% respectively in cases of pre-senile ATD compared to age-matched controls but there was a disproportionate reduction in HMW-SS ( $p < 0.05$ ). In cases of DS with the neuropathological and neurochemical changes of ATD, the total

concentration of SSLI did not significantly differ from control cases. Although SS14 was not decreased in DS, HMW-SS was significantly lowered by 58%. No change in total SSLI was observed when temporal cortex from senile ATD cases was compared to age matched controls. Additionally, the molecular forms of SSLI present in senile ATD were not significantly different from age matched controls.

The possibility that changes in cholinergic neurones in ATD and DS influence somatostatin-containing neurones to alter the processing of proSS was investigated (Chapter 6). In rat hippocampus ChAT activity was reduced by 89% two weeks after an electrolytic lesion of the septum. However, total SSLI and the proportions of the multiple forms of SSLI in the hippocampus remained unchanged. This finding shows that processing of proSS in the hippocampus in the rat is unaffected by removal of the cholinergic input to the structure and suggests that changes in the proportions of the multiple forms of SSLI observed in ATD are independent of an intact cholinergic input to the temporal cortex.

If the reduction in total SSLI seen in pre-senile ATD (Chapter 5) was due solely to a loss of somatostatin-containing neurones, the proportions of the molecular forms of SSLI would not be expected to change. The disproportionate reduction in HMW-SS observed in pre-senile ATD and DS is probably due to decreased biosynthesis of proSS which may be accompanied by an increased rate of post-translational processing. There is evidence to suggest increased ribonuclease activity in ATD (Sadjel-Sulkowska & Marotta 1984), which may reduce levels of SS-mRNA and also evidence suggesting that DNA repair is impaired in ATD (Robbins et al., 1983,

Li & Kaminskas, 1985). Other factors may also be important in regulating the synthesis and translation of SS-mRNA.

A damaged neurone in ATD may switch protein synthesis away from secreted proteins such as neurotransmitters, into structural protein or plaque and tangle protein. Changes in the expression of neuropeptide genes, which are not severe enough to alter total peptide levels, may be enough to alter the levels of prohormones or biosynthetic intermediates. Although the levels of most peptide hormones appear to be unchanged in ATD, investigation of the multiple forms of other peptide transmitters may show that their biosynthesis is altered in ATD.

In some brain regions, total SSLI levels are unchanged in ATD compared with age-matched controls. However, in these regions the proportions of multiple forms of SSLI have not been investigated. It will be interesting to establish whether or not HMW-SS is reduced in these areas, or whether a reduction only takes place in severely affected areas such as the temporal cortex, where total SSLI levels are reduced.

The studies reported in this thesis have shown that in the rat, a specific population of neurones projecting to the median eminence differs from neurones in other parts of the CNS in the pathway of post-translational processing of proSS. Recent neuroendocrine studies (Christie et al., 1986) have shown that levels of two pituitary hormones, GH and TSH, are elevated in ATD compared with age-matched controls. The inhibition of release of both of these peptides is regulated by somatostatin. Investigation of the multiple forms of SSLI present in the median eminence from human control subjects and from cases of ATD, may show that SS28

containing neurones respond differently from SS14 containing neurones to the proposed reduction in synthesis of proSS seen in ATD.

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